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INDUCTION OF BIMODAL PROGRAMMED CELL DEATH IN MALIGNANT CELLS BY BENZOPHENANTHRIDINE ALKALOIDS AND UKRAIN (NSC-631570)

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Abstract

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) and apoptosis are expected to provide new leads for safer, more effective chemotherapy. We have investigated whether the semisynthetic alkaloid-thiophosphoric acid derivative Ukrain (NSC-631570) could induce apoptosis in human K562 leukemia cells. Results showed that these alkaloids and Ukrain induced two distinct modalities of cell death programs. One modality corresponds morphologically to that of classical apoptosis 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 modality characterized by cell surface blister formation, high specific ^{51}Cr release and an apparent increase in cellular DNA content. These two cell death modalities 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.

Key Words: Alkaloids, apoptosis, blebbing, chemotherapy, immune effector cells, leukemia, programmed cell death, Ukrain.

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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 (biological response modifiers, 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 [8]. The latter event, i.e., internucleosomal DNA fragmentations, may not occur in some experimental systems [15]. Blebbing of the cells' surface membrane has consistently been found to occur during T-cell mediated tumor cell cytolysis [11], exposure to chemotherapeutic drugs [5], low temperature [9], anoxia [6], as well as during normal embryonic development [18]. It is thought that the death of normal cells is under the control of intrinsic genetic mechanisms which when properly executed, result in normal embryonic development [4], whereas failure to express the putative death genes may result in malignant cell growth [17]. It is estimated that there are about 100 oncogenes, and when some of them malfunction, they may stimulate cell growth and result in the development of malignant growth and cancer. Conversely, there are at least six tumor suppressor genes which are thought to oppose the expression of the malignant process [14].

From the practical point of cancer therapy, the ideal chemotherapeutic agent would be one that (a) triggers tumor 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, would still exert its tumor selective cytolytic activity.

Ukrain (NSC-613570) was screened *in vitro* by the National Cancer Institute (NCI, Bethesda, MD) Developmental Therapeutics Program and found to have cytotoxic effects on their 60 human tumor cell lines, representing eight tumor types [12]. Results of these assays showed that the mean molar concentration required to achieve 50% cell

growth inhibition were in the range of $10^{-5.5}$ M; 10^{-5} M for total cell growth arrest and $10^{-4.5}$ M for 50% loss of the initial cell biomass [12]. In view of this evidence, we set out to investigate whether Ukrain would trigger apoptosis in human malignant cells. For this purpose, we chose the K562 leukemia cell line, which had been found to be relatively resistant to the cytostatic and cytotoxic effects of these agents in the NCI-Developmental Therapeutics *in vitro* tests.

Materials and Methods

The K562 erythroleukemia cell line was purchased from the American Type Culture Collection (Rockville, MD) and maintained in culture according to the information accompanying the cell shipment (RPMI-1640 + 10% fetal bovine serum, FBS). Cells were passaged as necessary and maintained at 37°C, 5.0% CO_2. Ukrain (NSC 631570) $C_{66}H_{75}N_6O_{18}PS \bullet 6HCl$ was produced in pure crystallized form by J.W. Nowicky (Nowicky Pharma, Vienna, Austria). This agent was dissolved in water at a concentration of 1.0 mg/ml before use. Further dilutions, carried out shortly before *in vitro* use, were 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 96 flat bottom well plates. Triplicate wells of each drug concentration, containing 50,000 cells per well, were incubated at 37°C + 5.0% CO_2 for four hours. Morphological changes were monitored and quantitated by light microscopy at four hours of drug exposure. All experiments were repeated at least five times and morphological changes quantitated microscopically.

Membrane permeability changes

Cell membrane permeability changes were evaluated by $Na_2{}^{51}CrO_4$ release assay [7]. For this purpose, cells were labelled with 200 µCi of $Na_2{}^{51}CrO_4$ for 1-2 hours 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 the test compounds were evaluated in triplicate wells at 20,000 cells per well after four hours of incubation at 37°C in 5.0% CO_2. The percentage of ^{51}Cr release was calculated by the following formula (cpd = compound):

$$\%\ \text{specific}\ {}^{51}\text{Cr released} = \frac{(\text{cells + test cpd.}) - \text{spontaneous release}}{(\text{maximum}\ {}^{51}\text{release}) - \text{spontaneous release}} \times 100$$

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. These experiments were carried out in triplicate, repeated at least three times and statistically analyzed for $p < 0.05$.

Cellular DNA content assays

The DNA content of K562 cells exposed to various concentrations of Ukrain were analyzed using propidium iodide and flow cytometry. For this purpose, 0.5 x 10^6 K562 cells per well were aliquoted in 12 well plates and exposed to eight serial dilutions of each test compound 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 phosphate buffer saline (PBS) buffer containing 2.0% formaldehyde and cells were transferred to 12 mm x 75 mm tubes, pelleted by centrifugation and medium removed. Cell pellets were treated with 200 µl of ice cold ethanol for ten minutes, washed two times in PBS buffer and treated with RNAase (100 µg/ml) for 30 minutes 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 analyzed in a flow cytometer. These experiments were replicated at five times or more.

Results

The morphology of control K562 erythroleukemia cells is illustrated in Figure 1a. When these cells were exposed to various concentrations of Ukrain and periodically observed microscopically, it was found that at 2-4 hours, the classical morphology of apoptosis became manifested in ≥ 90% ± 4.5 of the cells at concentrations of 6.25 or 12.5 µg/ml drugs (Fig. 1b). Note that these cells displayed blebbing and shed closed right side out membrane vesicles from the cells surface. This morphology is consistent with current criteria of apoptosis [8].

The unexpected morphological finding occurred at a higher drug concentration, i.e., 25.0 µg/ml, where the morphology of apoptosis was no longer manifested. We refer to this drug concentration interval as the silent period (SP) based on the fact these cells displayed only slightly altered cell surface morphology similar to that of control cells except for loss of cell surface microvilli, not exposed to drug (Fig. 1c). However, at higher concentrations of Ukrain (50-100 µg/ml), we discovered that > 90% ± 3.5 of these cells displayed predominantly one or two large cell surface membrane blisters (Fig. 1d). Thus, Ukrain induced the classical morphology of apoptosis followed by a quiescent or silent period and subsequent blister formation on the cell surface membrane (Fig. 1d). The cell blisters consist of one large cell surface outpocketing where cell surface microvilli or small vestibular outpocketings are absent (cf.

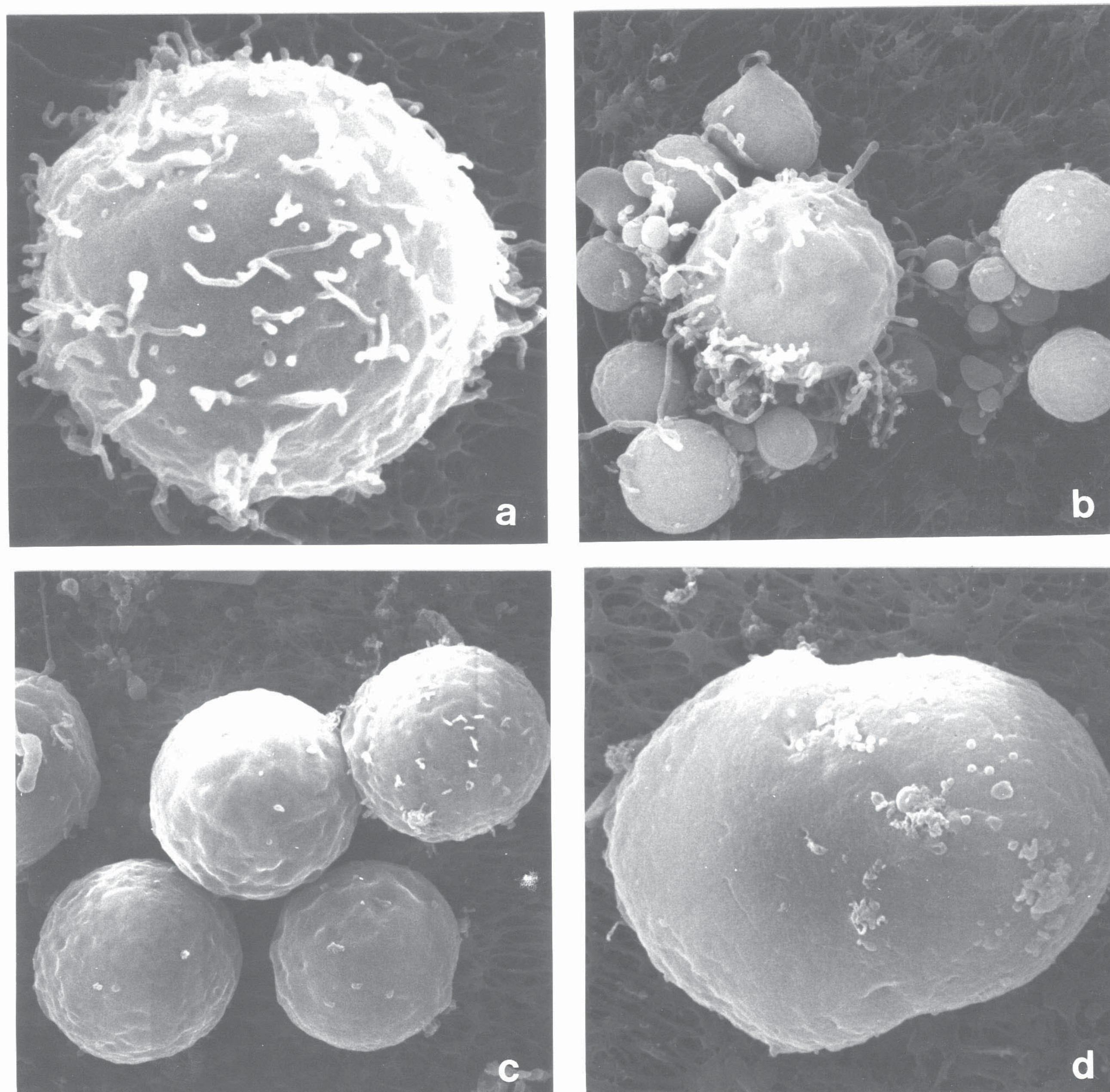

Figure 1. Effects of alkaloids on the morphology of human K562 cells. (**a**) Control K562 cells cultured for 4 hours without drug. (**b**) Cells cultured for 4 hours in the presence of 6.25 µg/ml of Ukrain display classical morphology of apoptosis, characterized by blebbing and shedding membrane vesicles from tumor cell surfaces; (**c**) K562 cells treated with 25 µg/ml of Ukrain enter the silent period characterized by relatively normal cell morphology, which corresponds to low, background levels of specific ^{51}Cr release (cf. Fig. 2). (**d**) K562 treated with 50.0 µg/ml of Ukrain display cell surface blister formation is associated with high specific ^{51}Cr release (cf. Fig. 2).

Fig. 1d). At the transmission electron microscopy (TEM) level, the cell blisters are devoid of major cytoplasmic organelles and contain primarily small ribosome-like particles (data not shown).

In order to establish the relationship between the apoptosis related cell morphology and the well established criteria of cell injury and death, we used the ^{51}Cr-release assay which is the standard criteria for immuno effector cell

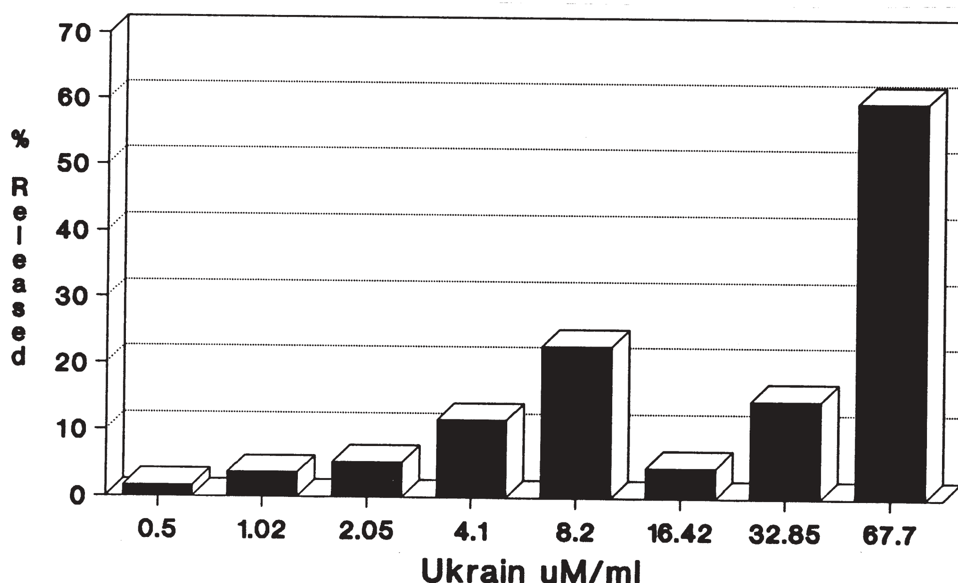

Figure 2. Bimodal specific ^{51}Cr release by K562 cells treated with various concentrations of Ukrain for 4 hours *in vitro*. Note that the first phase of ^{51}Cr release occurs at 4.1 and 8.2 µM of Ukrain; followed by background level of ^{51}Cr release at 16.4 µM (silent period); high specific ^{51}Cr release occurs at 67.7 µM which corresponds to cell surface blister formation (Fig. 1d).

lysis of tumor cells [7]. Results of the ^{51}Cr release assays showed an unexpected bimodal isotope release kinetics (Fig. 2). Significant ^{51}Cr release ($p \leq 0.05$) 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 25.0 µg/ml (Fig. 1c). The following two step increases of drugs (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 surface blister formation (Fig. 1d). Thus, a high fidelity was found between the bimodal morphological changes (Fig. 1) and the bimodal specific ^{51}Cr release kinetics of Figure 2.

DNA content of K562 cells treated with Ukrain was analyzed by standard agarose gel electrophoresis, as previously used in our laboratory [10]. 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 (Fig. 3). The cells did not show any morphological changes until the concentration of Ukrain reached 3.12 and 12.5, where cells displayed the classical morphology of apoptosis, 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, i.e., subdiploid DNA content (Fig. 3). Moreover, cells exposed to 25.0 µg/ml of Ukrain showed primarily loss of cell surface microvilli when studied at the SEM level (Fig. 1c). Further doubling in drug concentrations to 50.0 and 100.0 µg/ml produced an extensive apparent increase in cell DNA content (Fig. 3), with concomitant changes in cell morphology, which consisted of the formation of a single large cell surface blister (Fig. 1d). At the light microscope level, the blisters appear free of cytoplasmic organelles which was confirmed at the TEM level, where only ribonucleoprotein (RNP) particles were found present within this area (data not shown).

Discussion

Genetically programmed cell death is thought to underlie the morphology of apoptosis which has generally been found to correlate with nuclear DNA fragmentation [18] and is considered to be the biochemical hallmark of apoptosis [1, 3]. This DNA fragmentation is thought to occur at the internucleosomal regions due to the activation of a specific endonuclease [2]. Not all cells, however, manifest a strict correlation between the morphology of apoptosis and nuclear DNA fragmentation [16]. 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 hours [13]. At shorter time intervals, i.e., four hours, no significant K562 cell ploidy could be detected, whereas at 12 hours, an increase in cells at the G2 phase of the cell cycle was detected [13].

Contrary to the aforementioned studies, our data showed that Ukrain was highly effective in inducing an apparent increased cellular DNA content or polyploidy in K562 cells within 3-4 hours. As illustrated in Figure 3, Ukrain

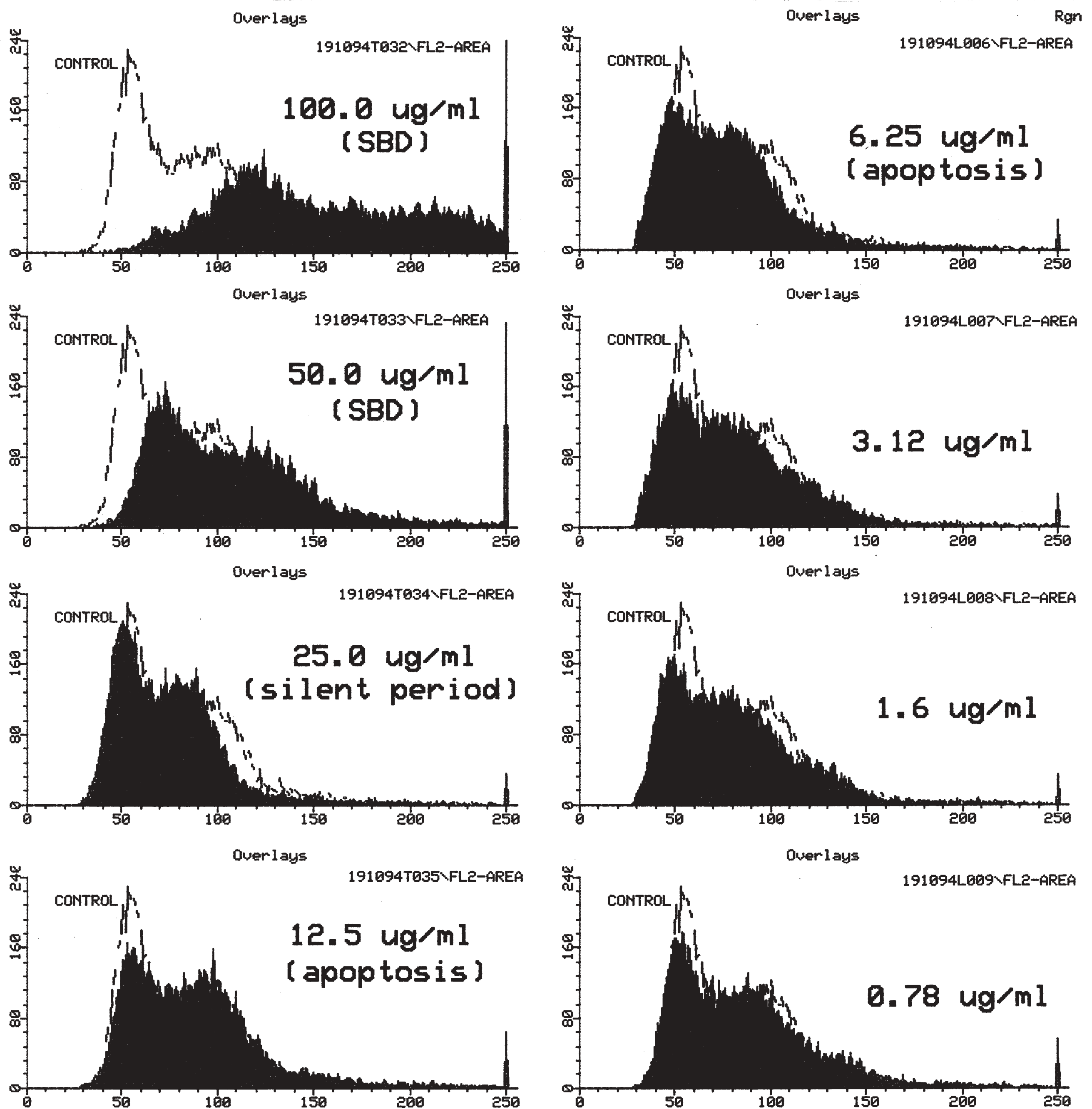

Figure 3. Effects of Ukrain on K562 cell DNA content measured by propidium iodide staining and flow cytometry. Note that at 67.7 and 32.8 µM of Ukrain for 4 hours, extensive DNA ploidy is induced. This increase in nuclear DNA content corresponds to cell surface blister formation (Fig. 1d) and high specific ^{51}Cr release (Fig. 2). Serial dilutions of Ukrain showed cells with normal diploid DNA content (from 16.4 down to 0.5 µM). Note that the morphology of the apoptosis or PCD stage (Fig. 1b) is in association with normal DNA content.

at concentrations of 50.0 µg/ml induced a practical disappearance of cells with G1 phase DNA content, by shifting the majority of them to a triploid or polyploid state of DNA content (Fig. 3). It is significant to note that these high levels of DNA content correlated with the cells morphology of 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 fidelity between cell morphological changes, high specific ^{51}Cr release and an apparent extensive increase in DNA content, all of which occurred in four hours or less of exposure to Ukrain. To our knowledge, there is no precedent 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 cycloheximide, 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 increase in DNA content was intranuclear.

Cell surface blister formation in hepacytes 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 bleb was considered by the authors to represent the abrupt transition from reversible to irreversible cell injury. This interpretation of the blister formation and eventual rupture is in agreement with our findings, where this cell morphology correlates with the loss of clonogenic potential of cells exposed to the alkaloid derivate Ukrain for only four hours. Thus, our findings are unique in that we have identified two distinct cell injury and potential death modalities induced by Ukrain in relatively short term culture conditions. The unique and novel finding, that polyploidy is associated with the second cell death modality, i.e., BCD, with concomitant high levels of specific ^{51}Cr release, strongly suggest 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, suggest 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 the alkaloid induces bimodal cell death programs, the first of which, i.e., apoptosis, is mediated by quinidine sensitive Ca^{2+} dependent K^+ channels [8] and the second modality, i.e., blister cell death, by preventing microtubule formation resulting in G2 cell cycle arrest, and thus resulting in an increased nuclear DNA content as measured by flow cytometry of propidium iodide stained cells. However, it is conceivable that the alkaloid derivative Ukrain induced extensive physical changes in DNA structure, such as unwinding of the double helical structure, and thus allowed a significant increase in propidium iodide binding. Clearly, future biochemical studies aimed at direct quantitation of DNA content per cell will be required to resolve this paradigm.

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Discussion with Reviewers

K.M. Anderson: Do the authors believe that DNA synthesis occurred or only a redistribution of the original DNA?
Authors: Transmission electron micrographs of cells that were treated with Ukrain and showed elevated DNA content did not display the characteristic chromatin condensation associated with apoptosis. Similarly direct observation of cells stained with propidium iodide for flow cytometry also showed uniform nucleoplasmic fluorescence without brighter areas that characterize DNA redistribution within the nucleus.

Thymidine incorporation experiments have not shown significantly higher DNA synthesis of Ukrain treated cells over that of controls. These results would indicate that the increased propidium iodide staining of treated cells is not due to conventional DNA synthesis mechanisms. However, it is conceivable that the increased propidium staining may be due to DNA repair mechanisms which may not depend on thymidine utilization.

Our current hypothesis for the apparent increased DNA content in Ukrain treated cells is that it may be due to structural changes, i.e., helix unwinding, allowing greater propidium iodide binding, and thus resulting in an apparent increase of DNA content per cell. We are currently investigating the possible mechanisms that may account for the dramatic increase in fluorescence and apparent increase in DNA content in cells undergoing alkaloid induced blister mediated cell death.

K.M. Anderson: Might treatment of cells with formaldehyde have cross-linked smaller DNA ensembles into larger entities that would register as “polyploid” nuclei on flow cytometry?
Authors: In preparing cells for flow cytometry, we have used alcohol fixations as well as formaldehyde. Both methods have consistently given the same result, i.e., apparent increase in DNA content in cells with blister morphology. Agarose gel electrophoresis of extracted DNA did not show DNA fragmentation during apoptosis, the silent period or the blister stage. Moreover, agarose gels of DNA extracted from equal numbers of cells have shown that cells in the blister stage yield a more intense staining (ethidium bromide) band than that of DNA extracted from cells in apoptosis and/or control untreated cells. However, since ethidium bromide is also a DNA intercalating fluorochrome, the apparent increased fluorescence may be also due to possible DNA unwind induced by the alkaloid derivative Ukrain.

Surprisingly, there are no sensitive DNA quantitation techniques that can distinguish DNA from bound heterocyclic compounds such as alkaloids, which absorb in the 260 µm ultraviolet range.