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K. M. Anderson
Rush Medical College

T. M. Seed
Argonne National Laboratory

J. Peng
Rush Medical College

A. Jajeh
Cook County Hospital

J. Meng
Rush Medical College

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Authors
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MORPHOLOGIC CHANGES OF APOPTOSIS INDUCED IN HUMAN CHRONIC MYELOGENOUS LEUKEMIA "BLAST" CELLS BY SC41661A (SEARLE), A SELECTIVE INHIBITOR OF 5-LIPOXYGENASE

K.M. Anderson[1,2,*], T.M. Seed[3], J. Peng[1], A. Jajeh[4], J. Meng[1], and J.E. Harris[1]

Departments of Medicine[1] and Biochemistry[2], Rush Medical College, Chicago, IL 60612
[3]Center for Mechanistic Biology & Biotechnology, Argonne National Laboratory, Argonne, IL 60439
[4]Department of Medicine, Cook County Hospital, Chicago, IL 60612

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Abstract

Several inhibitors of the arachidonic acid-metabolizing enzyme, 5-lipoxygenase reduce proliferation of hematopoietic and non-hematopoietic cells and cell lines and some cells undergo limited differentiation. Cells were cultured from patients with chronic myelogenous leukemia in "blast" crisis with the selective inhibitor of 5-lipoxygenase,SC41661A[3-{3,5-bis(1,1-dimethyl)-4-hydroxyphenyl}thiol]-N-methyl-N-[2-(2-phridinyl-propanamide)]. Cells cultured for 3 to 5 days with 40 μM SC41661A exhibited reduced cellular numbers along with ultrastructural changes and DNA laddering characteristic of apoptosis. Similar culture conditions reduced proliferation of U937 monoblastoid cells. In U937 cells, the ultrastructural features of apoptosis were not observed at 72 hours, when DNA laddering was present and cell numbers were reduced, but was present after 144 hours of culture. Dissociation between certain morphologic and biochemical sequelae of apoptosis has been described in other systems. These observations are of interest since the induction of apoptosis in dividing chronic myelogenous leukemia (CML) cells by a non-cytotoxic agent suggests paradigmatically new sites for therapeutic intervention.

Key Words: Leukemia, apoptosis, programmed cell death, 5-lipoxygenase inhibitor, SC41661A.

*Address for Correspondence:
Ken. M. Anderson
Section of Medical Oncology
Departments of Biochemistry and Medicine
Rush Medical College
Chicago, IL 60612, USA
Telephone number: 312-942-6423
FAX number: 312-942-3192

Introduction

Myelogenous leukemia cells from patients and the HL60 myelomonocytic and U937 monoblastoid cell lines express the arachidonic acid-metabolizing enzyme, 5-lipoxygenase [2]. This enzyme forms the hydroperoxy and hydroxy intermediates, 5-HPETE, 5-HETE, and leukotriene A4, leading to LTB4 (via LT4A hydrolase), LTC4 (by LTC4 synthetase) and LTD4 (due to gamma-glutamyl-peptidase), which can be subsequently cleaved by various dipeptidases to yield LTE4. While most of the biological effects of leukotrienes have been identified in studies of immune function and smooth muscle contraction, a role in modulating proliferation of at least some types of cells is a distinct possibility. Malignant cells from patients with chronic myelogenous leukemia synthesize excess amounts of leukotriene C4 [20].

Patients with myelogenous leukemia usually can be maintained for some time with several forms of therapy, but eventually the disease recurs and proliferating leukemia cells appear in the circulation. These cells are unresponsive to agents as employed previously and can be refractory to new therapy [9].

In the present study, we observed the induction *in vitro* of apoptosis in chronic myelogenous leukemia (CML) cells from patients in "blast" crisis by a selective non-cytotoxic inhibitor of 5-lipoxygenase [3, 10], SC41661A [3-{3,5-bis(1,1-dimethyl)-4-hydroxyphenyl} thiol]-N-methyl-N-[2-(2-phridinyl-propanamide)]associated with abortive attempts at expression of a differentiated function. While the mechanism of this response is unknown, the ability to access an apoptotic pathway inherent in myelogenous leukemia cells that have escaped first and often second-line therapy by a non-cytotoxic agent suggests future sites for therapeutic intervention.

Materials and Methods

Myelogenous leukemia cells from patients in blastic crisis or U937 monoblastoid cells were maintained in suspension culture by serial dilution every 3 to 4 days

Table 1. Effect of SC41661A on the 4 hour incorporation of ^{3}H-thymidine (cpm ± standard deviation, SD) into DNA of peripheral blood cells from patients with CML in blastic crisis and into U937 monoblastoid cells. N (number of samples) = 4 for each data point. Representative examples are presented. More extensive data are presented in references [3, 4].

	CML cells	U937 cells
Control	2063 ± 196	10406 ± 296
SC41661A		
20 μM	1743 ± 219	4728 ± 322
40 μM	1324 ± 386	3673 ± 640
80 μM	525 ± 40	233 ± 26

Table 2. The effect of 40 μM SC41661A on cell numbers after 5 days of culture (x 10^{-4}/ml). N = 2. Representative experiments are presented and more extensive data are presented in references [3, 4].

	CML cells	U937 cells
Control	1.25 ± 0.34	66.9 ± 9.2
SC41661A	0.04 ± 0.03	27.4 ± 6.8

with RPMI (Rosewell Park Memorial Institute, Buffalo, NY) 1640 medium containing 5% fetal bovine serum (FBS; U937) or 10% FBS (CML), 24 mM HEPES {N-(2-hydroxyethyl) piperazine-N' (2-ethylsulfonic acid)}, 2 mM glutamine, 50 μg/ml streptomycin and 50 units/ml penicillin [17]. Before culture, cells from patients were first purified on Ficoll gradients. Cell lines were treated in log phase with 40 μM SC41661A in dimethylsulfoxide (DMSO; 1 μl/ml medium) after a 1:2 split with medium for 24 hours; in some experiments, incorporation of ^{3}H-thymidine was measured after 4 hours, in others cells were cultured for an additional 48 to 120 hours before samples were taken for cell counts and DNA electrophoresis.

Transmission electron microscopy (TEM) was performed according to conventional procedures including fixation in glutaraldehyde (2.5% in cacodylate buffer, pH 7.4) containing 4% sucrose for 2 hours at room temperature [1]. Subsequently they were washed in buffer, post-fixed with 1% osmium tetroxide for 1 hour, rinsed in buffer, stained for 1 hour in uranyl acetate, dehydrated with increasing concentrations of ethanol and propylene oxide and embedded in epon/araldite. Grids were stained with Reynold's lead and uranyl acetate and sections examined with either a Siemens's 101 or a 100 CX TEM.

Figure 1. Control cells from a patient with CML in blastic crisis. Bar = 2 μm.

Figure 2. Apoptotic cells with extensive cytoplasmic vacuolation and several others with extensively condensed chromatin with little residual cytoplasm. One cell with marginated chromatin may represent a necrotic cell. Bar = 2 μm.

DNA was isolated with a DNA/RNA isolation kit (United States Biochemical Co, Cleveland, OH) according to the accompanying instructions. Cells were centrifuged, washed and lysed with lysis buffer. After adding the extraction buffer, the aqueous phase was transferred to another tube and sodium acetate and isopropanol used to precipitate DNA. DNA was resuspended in TE buffer and incubated with DNase-free RNase at 40°C for 1 hour. Samples (2-5 μg DNA each) were electrophoresed on 0.8% agarose gels containing ethidium bromide (0.5 μg/ml gel) at 70-90 volts for 1.5 to 2 hours, bands visualized with ultra-violet light and photographed with a Polaroid camera.

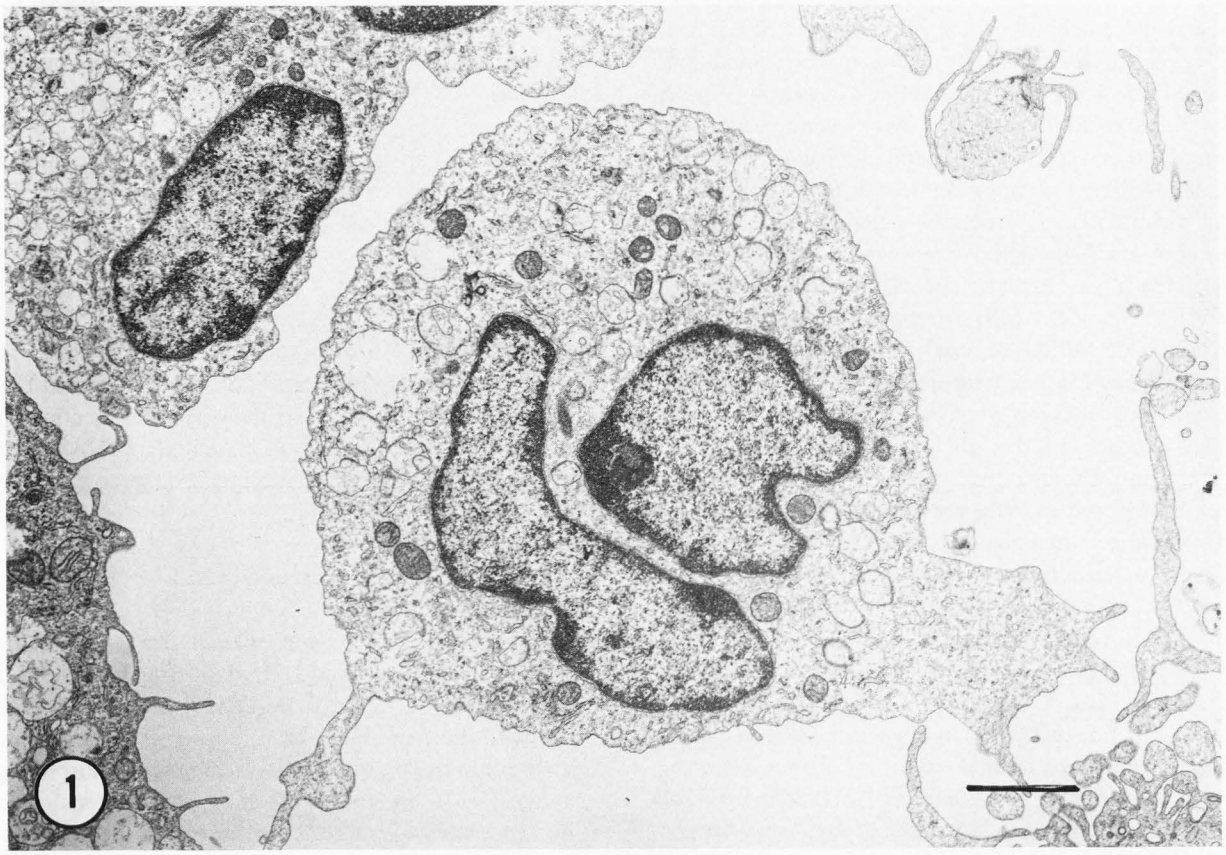
Work in progress employed an Apoptag kit from Oncor, Gaithersburg, VA.

Results

SC41661A reduced the proliferation of CML blast cells from patient's peripheral blood and of U937 monoblastoid cells in a concentration-dependent manner (Table 1). These results are typical of those reported in ref. [3, 4]; a lesser number of studies of the myeloblast HL60 cell line with similar results are in progress (results not shown). The concentration of SC41661A employed was 1 to 2 times the IC_{50} which inhibits PMN 5-lipoxygenase activity [15]. Uptake of trypan blue at 4 hour was unchanged in control and experimental groups (not shown). SC41661A is not considered to be cytotoxic [3, 4, 10, 15]. Cell counts after 3 or more days were greatly reduced in drug-treated cultures (Table 2; results are representative of additional experiments reported in ref. [3, 4]).

Aliquots of CML cells cultured for 5 days with SC41661A were removed for cell counts, viability and transmission electron microscopy. Over half the cells stained with trypan blue and cellular debris was present.

There was little evidence of apoptosis in control CML cells (Fig. 1) and only occasional (less than several percent) cells exhibited structural changes consistent with that process. SC41661A-treated cultures contained 50 percent or more cells exhibiting features of apoptosis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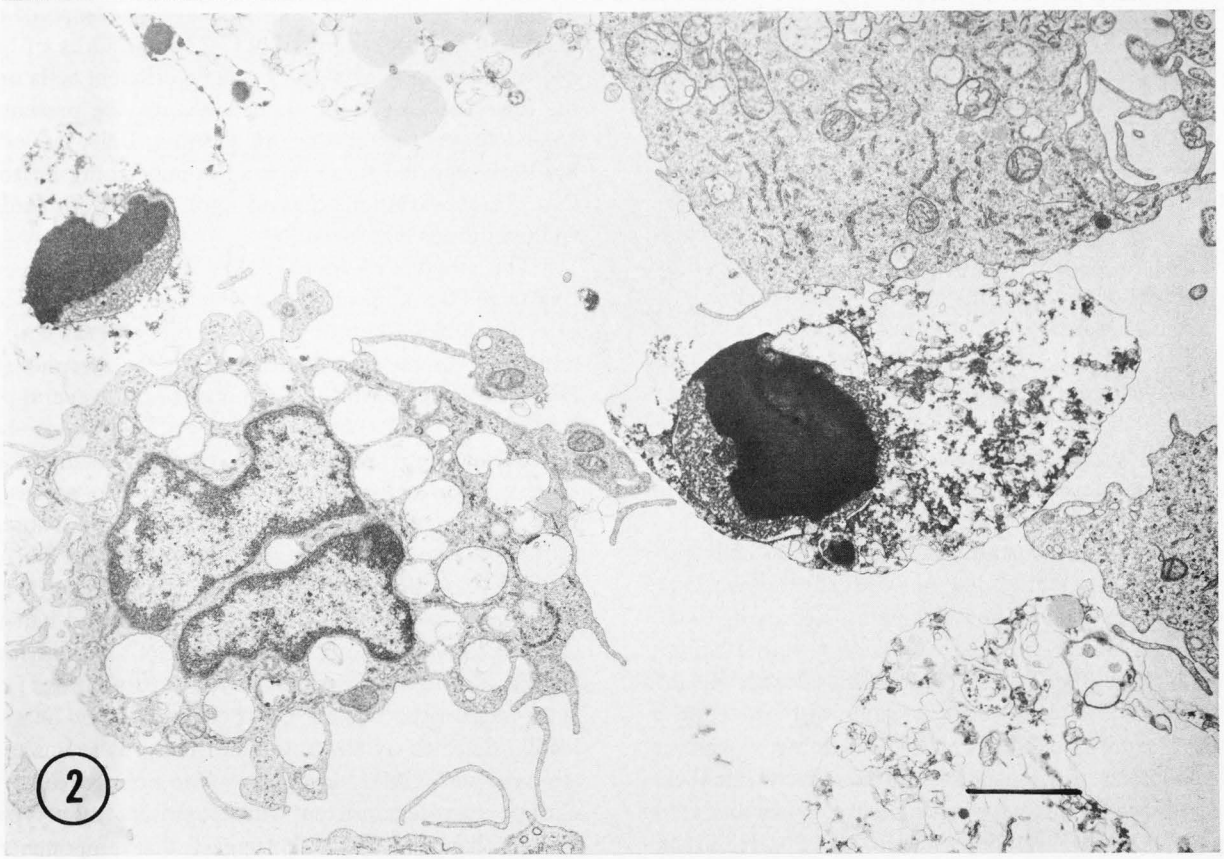
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(Figs. 2-7). These included shrunken cells containing typical nuclear apoptotic changes of condensed chromatin (Fig. 2) progressing to crescent-shaped accumulations in some cells and globular accretions in others (Figs. 3 and 4). Increasingly extensive cytoplasmic vacuolation occurred, some vacuoles representing what are interpreted as developing primary granules (Fig. 6). These represent differentiated structures consisting of vesicles containing inactive degradative enzymes related to immune defence and characteristic of more mature granulocytes [6]. In some cells, precipitated chromatin appeared to leak into the residual cytoplasm after dissolution of the nuclear membrane (Fig. 6), compared with other end-stage cells in which elements of chromatin remained attached to the nuclear membrane (Fig. 7). Generally mitochondria were well preserved until later stages of the process, when a variety of pycnotic fragments remained. In some cells, cytoplasmic vacuolation was more extensive than nuclear changes (Figs. 2 and 3). Either other regions of their nuclei with apoptotic changes were missed in the sectioning or such changes were absent. Other cells apparently remained unaffected by the drug (Figs. 4, 5, and 7). Many of the apoptotic cells were in the later stages of dissolution when changes of necrosis may be present.

One of a number of samples of CML cells from patients in blastic crisis cultured for 3 days with 40 μM SC41661A and examined for DNA laddering is presented in Figure 8A. Lower molecular weight multiples of DNA are present in this and in other comparable samples (not shown). We believe that laddering in the presence of SC41661A follows cessation of DNA synthesis, so that susceptible cells either were in or enter a proliferative stage of the cell cycle which is inhibited by the drug. Presently too few samples are available to definitely correlate the extent of proliferation as judged by the percentage of blast cells and DNA laddering.

Several ultrastructural studies of U937 cells cultured for 3 days with SC41661A have been done [e.g., 4]. Cytoplasmic or nuclear changes consistent with apoptosis were not seen. More recently U937 cells cultured with SC41661A for 120 hours were found to exhibit nuclear and cytoplasmic ultrastructural changes consistent with apoptosis (work in progress, not shown).

Although ultrastructural studies of U937 cells cultured for 72 hours provided no evidence of necrosis or apoptosis, DNA laddering was sought, employing 0.8% agarose gel electrophoresis and staining with ethidium bromide (Fig. 8B.). U937 cells cultured with 40 μM SC41661A for 3 days and compared with the DMSO (vehicle) control exhibited DNA laddering consistent with apoptosis in preparations in which cell numbers were reduced compared with controls. When analyzed with the Apoptag kit that identifies free 3'-OH termini

Figure 3. An apoptotic cell with crescent-shaped condensed chromatin, another cell with extensive cytoplasmic vacuolation and little nuclear change evident. Bar = 2 μm.

Figure 4. An apoptotic cells with extensively condensed chromatin, portions of which appear to be invaded by cytoplasmic vacuoles. Bar = 2 μm.

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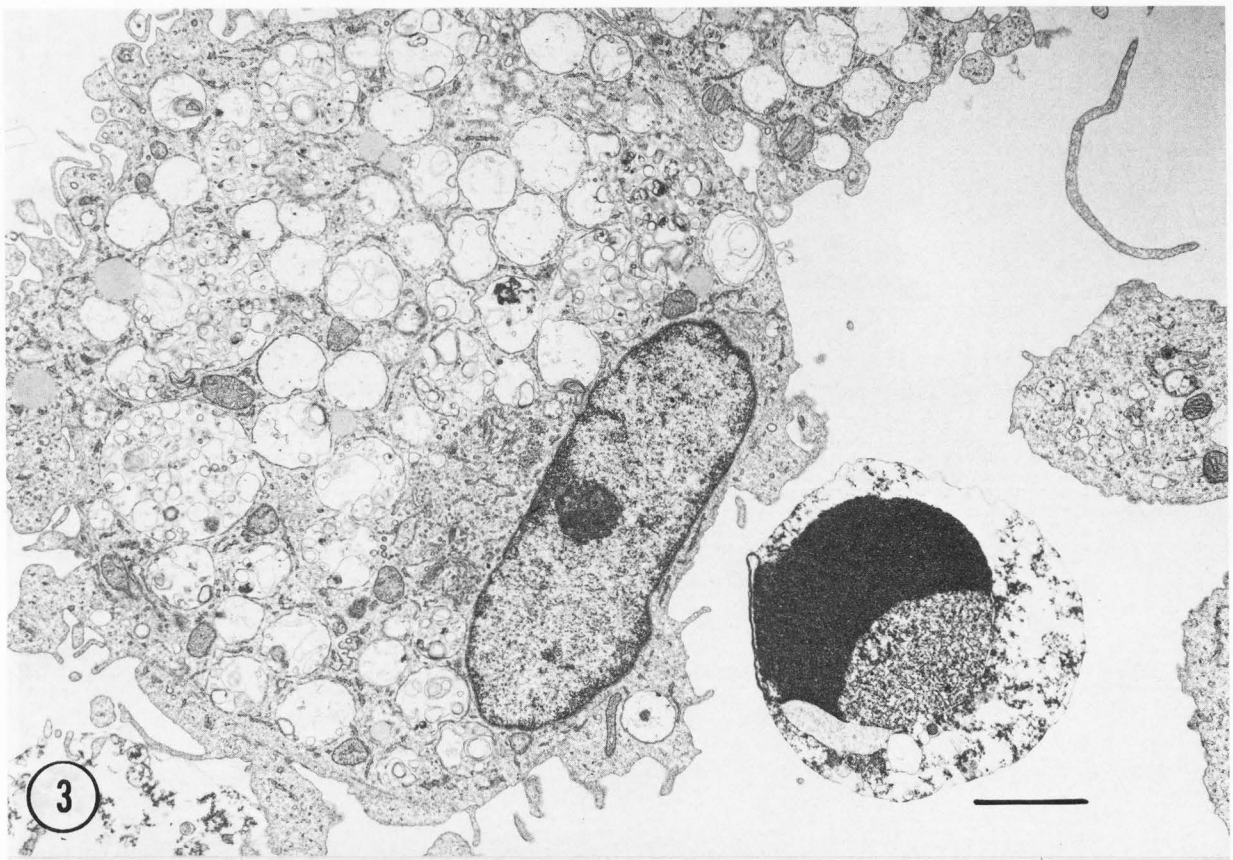
of DNA, intense brown staining indicating these free termini was present in nuclei of SC41661A-treated U937 cells with lesser staining of their cytoplasm, consistent with the presence of DNA at that site (not shown, work in progress). These results will be presented in a subsequent communication.

Discussion

Discrepancies between characteristic morphologic changes of apoptosis and DNA laddering have been described [7, 8, 11, 13]. If DNA degradation is not extensive with the generation of oligosomes, laddering may be absent in the presence of typical nuclear morphologic changes [13]. In studies of embryonic systems, extensive cytoplasmic changes consistent with apoptosis have been noted in the absence or a delayed presence of nuclear chromatin changes [7, 11, 22]. Features of both necrosis and apoptosis may occur in different cells or in the same cells and necrotic changes may be present as an end stage of apoptosis. In addition, DNA laddering has been reported to occur in cells undergoing necrosis [8]. These variations depend upon the system studied and conditions employed [5].

The kinetics of apoptosis, in some systems occurring from 20 minutes to perhaps an hour and the percent of cells involved before or during this interval will determine if apoptotic cells are detected by morphology, DNA laddering, or both. Apoptosis of only several percent of cells in a population can be sufficient to produce DNA laddering. Whether cells from all or only some stages of the cell cycle can be induced to access an apoptotic program also depends upon the system studied [16]. The rate at which high molecular weight DNA is degraded to oligomers should determine the ability to detect apoptosis by these means. A prolonged half-life of "nicked" high molecular weight DNA should favor "capping" or other large scale DNA accumulations [13], and characteristic nuclear DNA changes should be more readily detected by electron microscopy, as seems to be the case with CML blast cells. The presence of cytoplasmic changes consistent with apoptosis without typical nuclear changes [7, 11, 22] suggests that components of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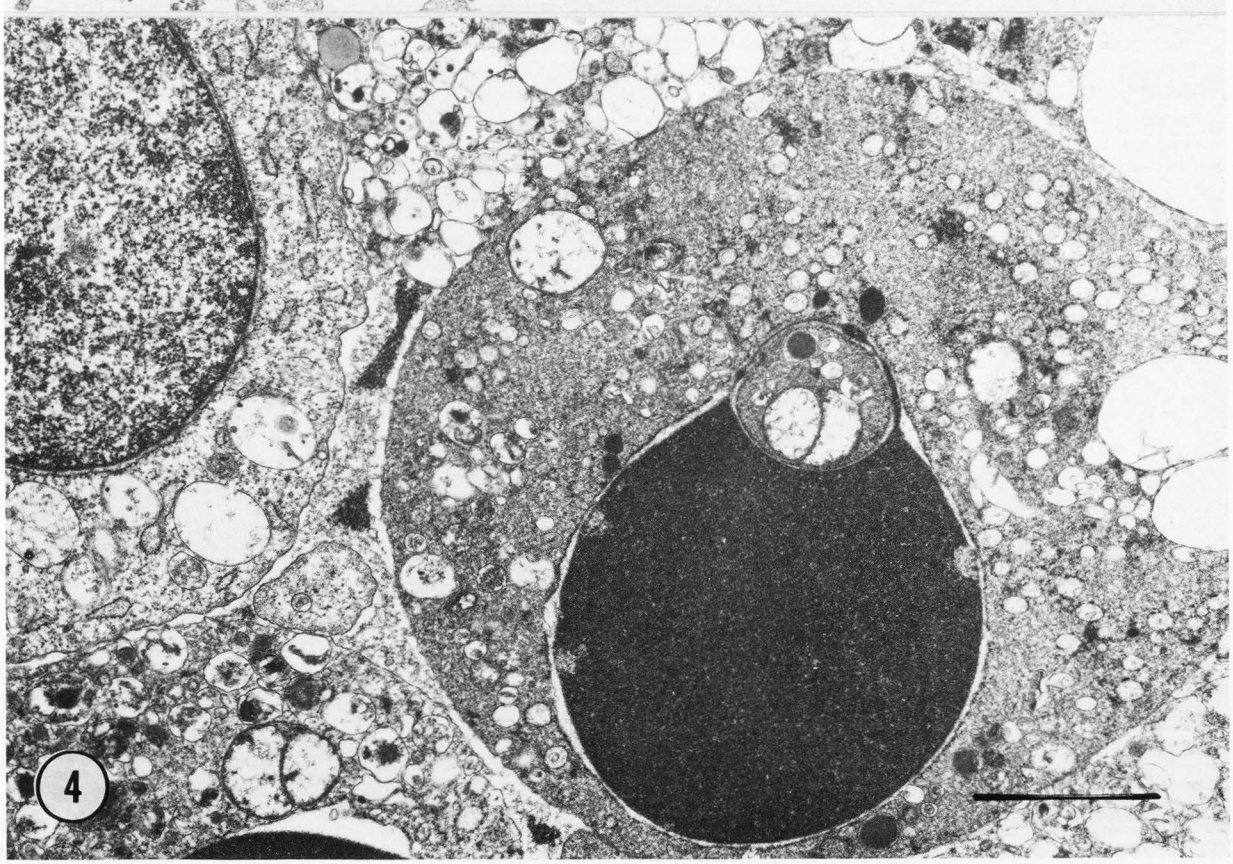
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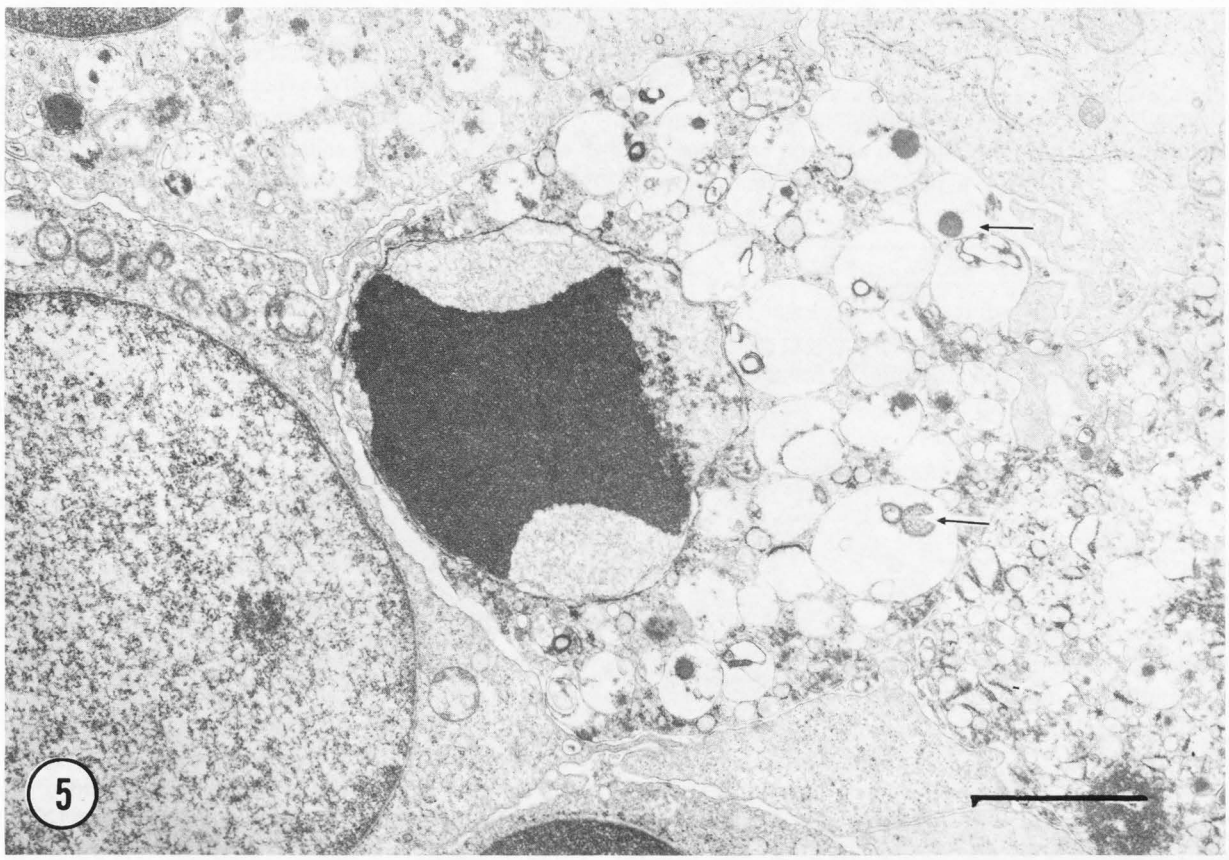

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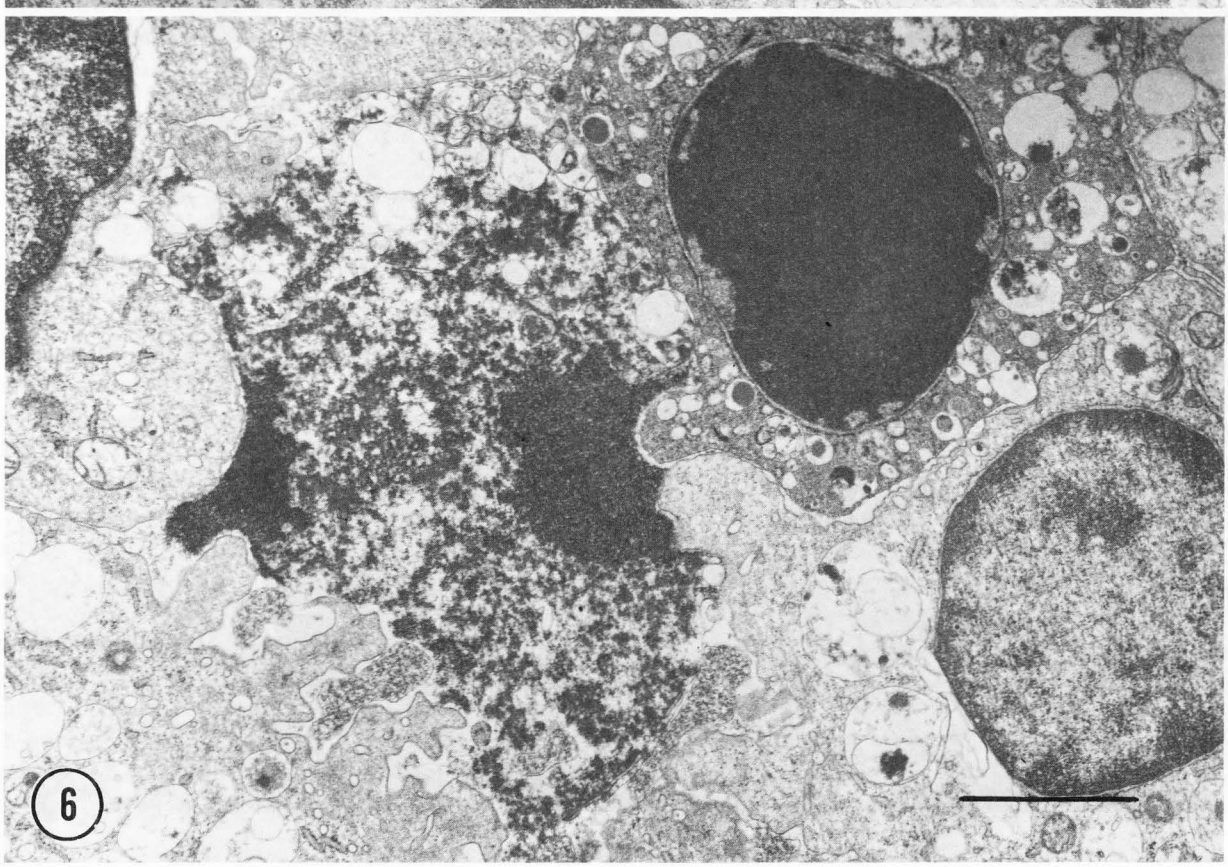

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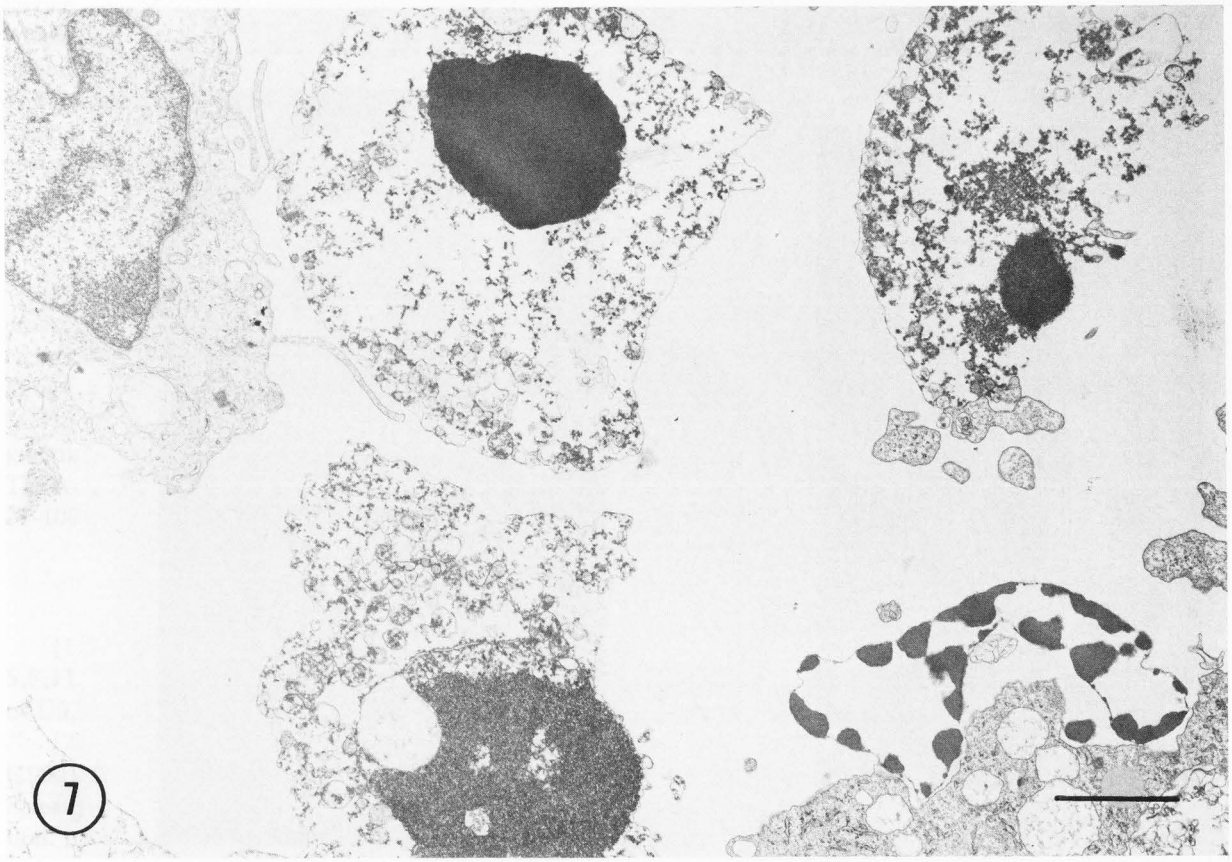

Figure 7 (above). End stage of apoptotic cell dissolution with residual nuclear chromatin bodies. Bar = 2 μm.

Figure 5 (on the facing page). Apoptotic cells with what appear to be swollen developing primary granules, a measure of differentiation. Bar = 2 μm.

Figure 6 (on the facing page). Apparent dissolution of the nuclear membrane with release of condensed chromatin into the remaining cytoplasm. Bar = 2 μm.

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the apoptosis "program" can be dissociated and individually activated. Some of these variations may depend upon karyotypic changes in the leukemia cell population, with development of progressively more aneuploid chromosomes determining some of their phenotypes. If during apoptosis affected cells exhibit some indeterminate marginated clumping of chromatin, swelling of the cytoplasm and its organelles with flocculent densities appearing within mitochondria, these changes would indicate accompanying necrosis which could confound the interpretation.

Most patients in blastic crisis have been on a maintenance therapy that has become unable to control the disease. Depending upon circumstances, the sample provided by the clinician may have been drawn before institution of new and possibly more successful therapy, or at some time thereafter. Following chemotherapy or radiation, apoptosis is a major mechanism of cell killing and apoptotic cells could be present in the control samples from effectively treated patients. These factors can complicate interpretation of these studies and may be the reason for some DNA laddering in control samples. An absence of actively proliferating cells may account for any lack of DNA laddering in drug-treated cultured cells, but this point needs to be examined with a larger number of samples containing differing percentages of replicating CML cells.

We now take up the issue of a proposed mechanism for these results. Do we believe that inhibition of 5-lipoxygenase by concentrations of a selective inhibitor that are 1 to 2 times the IC_{50} of the enzyme in mature granulocytes is responsible? It can be suggested that one or more products of 5-LP0x serve as an autocrine or paracrine growth factor, thereby expressing anti-apoptotic activity, or modulate the activity of such agents. There are precedents for this, since leukotriene products

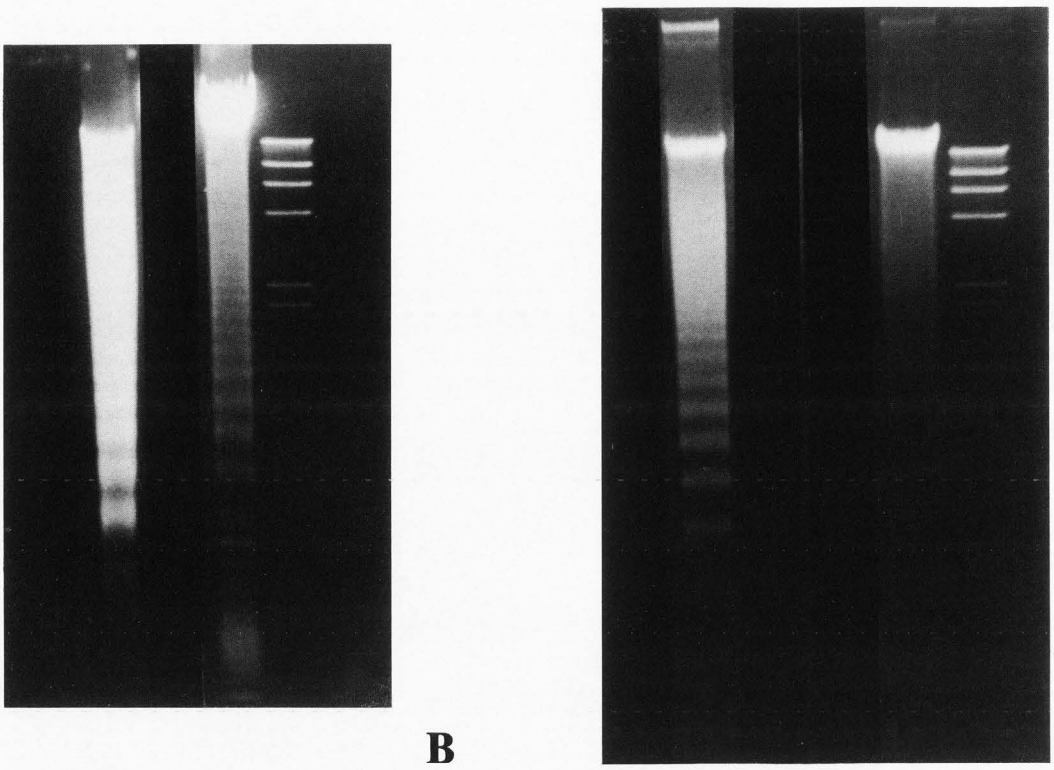

Figure 8. DNA laddering of CML blast cells (**A, at left**) and of U937 cells (**B, at right**) cultured with 40 μM SC41661A for 48 and 120 hours, respectively. D: DMSO control, middle lanes; SC: SC41661A, left lanes; DNA standard: lambda Hin dIII from 23.1 to 0.6 kB (faint lower band), right lanes.

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have been reported to serve the latter function [reviewed in ref. 2]. However, suramin, employed in the treatment of metastatic prostate cancer provides an instructive cautionary example. Originally reported to inhibit the interaction between epidermal growth factor and its receptor, more recently it was also shown to impair mitochondrial oxidative metabolism, providing a dual inhibitory effect on cellular proliferation [18]. It would not be surprising if besides inhibiting 5-LP0x, which is expected at the concentrations of SC41661A we employ [15], additional biochemical events may occur that contribute to suppression of proliferation and induction of apoptosis [15]. Originally Wickremasinghe *et al.* [21] reported that MK866, a unique inhibitor of 5-LP0x "tethering" to membranes (required for enzyme function) did not reduce proliferation of cells known to contain 5-LP0x, while other inhibitors of the enzyme that included oxidation/reduction agents were able to inhibit proliferation of cells in which 5-LP0x was considered to be absent. However, subsequently they reported that MK866 inhibited proliferation of CML cells from 40% of the patient samples studied, indicating that replication of CML cells in this large subset was dependent upon functional 5-LP0x activity [14].

It is certainly possible that SC41661A at 40 μM inhibits this subset of replicating CML cells because of its anti 5-LP0x activity, but in addition reduces the rate of proliferation of the other 60 percent of CML cells by additional mechanisms yet to be identified. Cells apparently unaffected by the drug may not have been cycling, which may be a prerequisite for its channelling of the cells into apoptosis. Antitumor effects of 5-LP0x have been examined in at least two *in vivo* studies. Nafazatrom (Bay q 6575) (at 0.8 mg/kg) inhibited the growth of B16 melanoma cells in nude mice by 90 percent [12], while SC41661A at 1.15 mg/mouse i.p. protected nude mice from OVCAR human ovarian carcinoma cells [10] without cytotoxic effects on the animals. SCID (severe combined immunodeficient) mice that receive human CML cells intravenously exhibit a disease mimicking

human leukemia and may represent a suitable model in which to test any antileukemic effects of 5-LP0x inhibitors [19]. Whether SC41661A or other inhibitors of 5-LP0x are active *in vivo*, they may sensitize leukemia or other malignantly transformed cells to chemotherapy, biological response modifiers or radiation therapy.

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Discussion with Reviewers

A. Liepins: In the text you say: "SC41661A-treated cultures contained 50 percent or more cells exhibiting features of apoptosis (Figs. 2-7)"; what are the "features of apoptosis"?
Authors: Generally, it is agreed that cells undergoing apoptosis decrease in size, nuclear changes of unusual condensations of chromatin occur, followed by internucleosomal scission and release of oligomeric nucleosomal arrays. Cytoplasmic "apoptotic" vesicles appear but generally, organelles such as mitochondria are relatively unaffected until later in the process. Phagocytosis of affected cells and cell remnants occurs *in vivo*; *in vitro*, without exposure to scavenger cells, disruption also occurs with release of vesicular material at times containing residual nuclear chromatin. As mentioned below, programmed cell death occurring in embryonic systems need not express all of these features [7, 8, 11, 13, 22].

A. Liepins: In the text you also say: "Increasingly extensive cytoplasmic vacuolation occurred, some vacuoles representing what are interpreted as developing primary granules (Fig. 6)"; what are "primary granules"?
Authors: "Primary granules" normally begin to appear in the cytoplasm of leukocytes during very early differentiation, and structures that fulfill this description were seen in SC41661A-treated CML cells. When fully differentiated, such bodies contain various degradative enzymes used in the host defence against bacteria and other engulfed foreign bodies. Please see reference [6] for further information about these structures.

E. Falcieri: Why did you use 4% sucrose in the fixative? Can you exclude an osmotic effect on the cell?
Authors: Use of 4% sucrose in the fixative is intended to avoid osmotic effects and is a fairly standard technique in a number of laboratories.

E. Falcieri: It is well known that frequently the apoptotical process is followed by a "secondary necrosis". This is characterized by a progressive hydration of cytoplasm and organelles, with initially minimal nuclear involvement. Plasma membrane and organelle disruption successively appear, allowing the lysosomal content to leak out into the cytoplasm. In the apoptotic cell undergoing necrosis, only apoptotic nucleus can be recognized in the final stage. If assumed that apoptotic cells are characterized by strongly clustered or condensed chromatin, in most images presented in the paper late apoptotic features appear. How could the authors explain this behaviour? Why did you not see typical "early" apoptotic patterns long described in the literature?
Authors: We agree that many of the apoptotic changes in the CML cells are relatively late; the most likely reason why we did not see early changes was the choice of 120 hours of drug treatment before isolation of cells for electron microscopy. It may be that accession of apoptosis is cell cycle-dependent and cells are only gradually accumulated in the appropriate (S?) phase of the cycle prior to "exit" into apoptosis. This answer to this question is being studied with U937 cells. The use of CML blast cells is more difficult due to (**a**) the relative infrequency of samples, and (**b**) requirement for ancillary growth factors such as IL3 and GM-CSF to achieve maximum *in vitro* cell replication which, due to their cost were not used.

The distinction between middle to late apoptosis and its end-stage when secondary necrosis can be present is not always easy to make. This is why, ideally, as many distinct features of the process, morphologic, biochemical and cell-biological/functional, as can be adduced to support the claim should be accumulated. The concept of apoptosis, whether it is distinct from programmed cell death, the appearance of what seems to be laddering of DNA from cells induced to undergo necrosis [8], the separation of DNA events in apoptosis from attempts at conservative DNA replication, and DNA repair reactions, have not been studied extensively, at least not all in the same system. Whether induction of apoptosis is cell type-dependent, inducible from all or only from certain stages of the cell cycle, which protooncogenes, P53, bcl-2, C-myc, C-fos, the RB gene participate in the various systems studied have not been coordinated into a common scheme. It is also true that cells induced to undergo necrosis by several means can exhibit "classic" DNA laddering [8]. For these and other reasons, a "final" common definition of "apoptosis" has not yet been developed. Since considerable variation among afferent signalling systems have been and, possibly less likely the efferent effector pathway(s) will be found, differences in detail among various pre and post-embryonic types of cells may preclude a common definition.

E. Falcieri: How do you interpret those cells with cytoplasmic alterations but without evident nuclear changes?
Authors: A number of cells exhibited cytoplasmic changes without evident nuclear alterations. That may have been due to sampling and the manner in which cells and their nuclei were sectioned. Alternately, in some aberrant transformed cancer cells, the agent em-

ployed might activate pre or post-nuclear events which bypass that organelle, leading to "downstream" cytoplasmic apoptotic events, as suggested to occur in certain forms of programmed cell death without early nuclear changes. Conceivably, the karyotypic changes in chronic leukemic cells undergoing blastic crisis "uncouple" some of the nuclear and cytoplasmic events observed in apoptosis of non-transformed cells with normal karyotypes. Possibly relatively undifferentiated cells utilize a "programmed cell death" pathway more akin to cell death occurring in some embryonic systems. This is an interesting question worth more thought.

M. Rubenstein: No biochemical markers such as the amount of bcl-2 protein or mRNA are used, nor is end group analysis using 3' terminal transferase labelling of fragmented DNA.
Authors: Bcl-2 protein is not a general "marker" for apoptosis, and for some types of cells, does not seem to be involved in the process. In other systems, it may retard or even prevent the process, but it is not thought to be a universally active antiiapoptotic agent in all types of cells. Studies with an end-labelling kit have been performed and will be reported separately.

J.A. Kellen: ... However, in order to consider *in vivo* studies, the effect of SC41661A could (and should) be tested by flow cytometry of control and treated cell cultures (... such as in Telford *et al.*, J. Immunolog. Methods, **172**, 1-16, 1994).
Authors: Flow cytometry is certainly another means of measuring DNA degradation. For some studies, especially those of the cell cycle susceptibility to apoptosis-inducing agents, use of the technique is ideal and such studies are in progress with U937 cells.

J.A. Kellen: Perhaps an indication as to the mechanism(s) of multiple drug resistance (MDR) would be appropriate in the **Introduction.**
Authors: We do not believe that discussion of multiple drug resistance is indicated for this paper. Resistance to drug-induced apoptosis, as distinct from drug resistance due to MDR may represent one of the numerous forms of "drug resistance". No studies of MDR1 protein or RNA synthesis were performed. Reasons why apoptosis is readily induced in some and not in other types of cancer is a largely unknown area of study, since the afferent and efferent signal transduction pathways and effector molecules for these events often differ among systems studied and generally are not well defined.

J.A. Kellen: In the text you say "There was little evidence of apoptosis in control CML cells (Fig. 1) ..." - by EM! Obviously, there must be some apoptosis, but was just not evident in the sample studied. Is less than 5% consistent with generally accepted numbers in leukemic cell cultures.
Authors: In control samples, we did not find a significant number of morphologically apoptotic cells (i.e., more than several percent, a "rare" cell was seen). Faint DNA laddering could at times be seen in some cultured control samples but less than that from treated samples exhibiting reduced numbers of cells. It has been reported that several percent of apoptotic cells in a population is sufficient to yield DNA banding. In other words, it depends upon the clinical setting and *a priori*, no rules are guaranteed. If patients have recently been effectively treated, significant apoptosis might be expected; if they are in true blastic crisis with major proliferation of blast forms, spontaneously apoptotic cells may be "lost" among the dividing forms unless their frequency is augmented by treatment. Non-dividing CML cells may not be susceptible to SC41661A-induced apoptosis.

J.A. Kellen: In connection with Table 2 you say in the text: "... results are representative ...". What is "representative"? Are these electron micrographs of the most characteristic and typical areas and samples or are they a typical average findings (from how many scans)?
Authors: "Representative" electron photomicrographs include areas from apoptotic cells selected by an experienced electron microscopist that characterized the majority of the apoptotic cells observed. As mentioned, other cells did not exhibit typical nuclear changes while presenting cytoplasmic findings consistent with apoptosis and a few cells appeared to be unaffected by the drug (discussed above). Whether the former represents programmed cell death in which extensive nuclear changes are not present, at least not early in the process, is unclear. Cells that appeared to be unaffected by the agent may never have been in cell cycle, which we believe but have not demonstrated, may be a requirement for SC41661A-induced CML cell apoptosis. Possibly malignantly transformed CML cells, in which normal differentiation is "blocked", express a more embryonic form of "programmed" cell death as a dysfunctional phenotype, as suggested above.

I.D. Bowen: The authors should note that other investigators have discovered inconsistency between the morphological symptoms of apoptosis and the incidence of DNA ladders (Bursch *et al.*, 1992; Zakeri *et al.*, 1993) to the extent that DNA laddering is being questioned as being diagnostic for apoptosis (Bowen, 1993).
Authors: The papers of Collins *et al.* (1992), Gershenson and Rotello (1992), Bowen (1993), and Zakeri *et al.* (1993) discuss these more recent findings and their interpretation. Apoptosis or programmed cell death in can-

cer cells may present special problems of interpretation.

Malignantly transformed cells such as CML or U937 cells in some sense "revert" to more (or are developmentally incapable of maturing from) "primitive" differentiation states in which apoptosis may not recapitulate the typical findings reported for mature cycling or terminally differentiated cells. Programmed cell death without typical DNA laddering (or with delayed laddering) may represent an earlier "precursor" form of non-necrotic cell destruction in developmentally arrested malignantly transformed cells. Whether various afferent or efferent pathways involved in either process can be accessed by non-cytotoxic means could be of considerable interest to those wanting to modify the biology of cancer cells.

J.A. Kellen: In Table 2, surely "(x 10^{-4}/ml)" means 10,000 cells/ml and should read 10^4/ml.

Authors: In the biochemical literature, a value of 10,000 generally is represented in tabular form as, e.g., (cpm x 10^{-4}). Thus, 12.0 stands for 120,000 x 10^{-4} = 12.0.

Additional References

Bursch W, Oberhammer F, Schulte-Hermann R (1992). Cell death by apoptosis and its protective role against disease. TIPS **13**, 245-251.