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# 9-nitro-camptothecin delays growth of U-937 leukemia tumors in nude mice and is cytotoxic or cytostatic for human myelomonocytic leukemia lines *in vitro*

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Abstract: The camptothecin derivatives 9-nitro-camptothecin (9NC) and 9-amino-camptothecin (9AC) inhibit similarly growth of HL-60, KG-1, and U-937 cells *in vitro*, whereas growth of THP-1 cells is inhibited by 9AC, but not by 9NC. Growth inhibition is accompanied by enlargement of cells which contain one (HL-60, THP-1) or more (KG-1, U-937) nuclei. Flow cytometry studies showed that 9NC-treated HL-60 and U-937 cells accumulate in the S and $G_2$ phases of the cell cycle; then they die by apoptosis, with the HL-60 cells being more sensitive than U-937 cells to 9NC. In contrast, 9NC-treated KG-1 and THP-1 cells accumulate in S and $G_2$ phases, but resist death by apoptosis. Of the cell lines tested, only U-937 cells xenografted in nude mice generated subcutaneous myeloid tumors, which exhibited a delayed growth in the presence of 9NC. Further, 9NC-treated advanced U-937 tumors regressed temporarily, indicating that U-937 cells consist of 9NC-sensitive and 9NC-resistant populations.

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Key words: camptothecin – myeloid cells – leukemia tumors – nude mouse – cell cycle

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## Background

Camptothecin (CPT) is a plant alkaloid that demonstrated high antiproliferative and toxic activity against the murine leukemia L1210 cells during studies concluded in 1966 (1). In the 1970s, a water-soluble form of CPT, Na-CPT, was used to treat patients with cancer in clinical trials, but low antitumor activity combined with severe toxicity led to its discontinuation (2–5). Subsequently, it was discovered that opening of the lactone ring of the CPT molecule in aqueous environment is accompanied by loss of the biological (anticancer) activity of the drug (6). This knowledge has prompted attempts to synthesize *de novo* or semisynthesize CPT derivatives that retain antineoplastic activity in an aqueous environment. Recently, one such CPT derivative, CPT-11, has been used in clinical studies on patients with refractory leukemias and lymphomas (7), non-small cell carcinomas of the lung (8), and gynecological cancers (9). The results of these studies have shown that CPT-11 has significant but limited anticancer activity. Moreover, CPT-11 has very little activity on tumor cells *in vitro*, and must be converted intracellularly to its metabolite SN-38 in order to exhibit an antiproliferative effect (10, 11). We have recently demonstrated that various CPT derivatives exhibit enhanced antitumor activity when used in suspension and administered intramuscularly or orally instead of intravenously (12; and unpublished data). This combination of appropriate vehicle and mode of administration has potentiated CPT and derivatives to effectively inhibit growth of several solid human tumors xenografted in nude mice, including colon, lung, breast, and malignant melanoma (12–15). Further, these drugs induce regression of advanced human malignant melanoma tumors grown in nude mice (15). Remarkably, CPT and derivatives are not toxic to nude mice, including those bearing tumor xenografts (15), at doses which are tumor-suppressing, an important observation from the biological, pharmacological, and clinical viewpoint. The mechanism(s) by which CPT and derivatives exert their selective antitumor activity is not yet understood. However, it has been documented that these drugs interfere with the topoisomerase I-mediated cellular process of breakage/reunion of chromosomal DNA (16–18). Specifically, topoi-

somerase I-generated DNA fragments form a complex in the presence of CPT consisting of topoisomerase I-DNA-camptothecin (reviewed in ref. 19). This complex, termed the cleavable-complex, cannot be utilized further in the reunion reaction, and therefore it is the limiting step in the breakage/reunion process (19). It was initially suggested that the quantity of topoisomerase I correlates positively with the CPT quantity, that is, tumor cells contain more enzyme than normal cells, and that CPT-resistant tumor cells express decreased levels of topoisomerase I (13, 14, 20–22). However, recent reports have shown that CPT-resistant cell lines synthesize normal levels or a mutated form of topoisomerase I, suggesting that differences in CPT activity may be associated with different forms of DNA topoisomerase I (23, 24).

An early event that takes place in human leukemia U-937 cells treated with CPT derivatives is the temporal upregulation of expression of the early response genes c-*jun*, *jun*-B, and c-*fos* (25). This is followed by induction of synthesis of the cytoskeletal protein vimentin, and other proteins associated with monocytic differentiation of U-937 cells (26). These findings have been correlated with internucleosomal DNA fragmentation in these cells (25, 26), a characteristic event that is induced by an endonucleolytic activity during programmed cell death or apoptosis (27). Of interest, the induction of early response gene expression and DNA fragmentation preceded the marked growth inhibition of U-937 cells, and enlargement of these cells, their nuclei and nucleoli (25). Flow cytometry studies on the cell cycle of human leukemia cells treated with CPT *in vitro* showed DNA degradation in S phase or cell-arrest in S and $G_2$ phases, depending on whether the leukemia cells were of myeloid or lymphoid lineage (26, 28–31). In this report, we describe 9NC-induced responses of human leukemia cell lines that represent cells at various stages of myelomonocytic differentiation. The responses include effects on proliferation, morphology, and distribution of cell cycle fractions. We further demonstrate that 9NC slows, but does not completely inhibit, the growth of human tumors generated by U-937 cells xenografted in nude mice.

## Material and methods

### Camptothecin derivatives

9-nitro-20(S)-camptothecin (9NC), 12-nitro-20(S)-camptothecin (12NC), and 9-amino-20(S)-camptothecin (9AC) were prepared in our laboratory from CPT according to a published procedure (32). Briefly, CPT was obtained from the Institute of Materia Medica (Shanghai, China) and purified further by chromatography methods (33). Purified CPT had a minimum purity of 99% as assessed by high-pressure liquid chromatography (HPLC). The experimental conditions for HPLC have been described (33). Further, 9NC, 12NC and 9AC were derived from purified CPT (32). For the *in vitro* studies, 9NC was suspended in polyethylene glycol (PEG 400; Aldrich, Milwaukee, WI) at 10 or 100 μg/ml, divided into small aliquots, and stored at −70 °C until used. For the *in vivo* studies, 9NC was used as a fine suspension in Intralipid 20% (KabiVitrum, Inc., Franklin, OH) or cottonseed oil (Sigma). For these studies, the drug suspensions were prepared under sterile conditions shortly prior to injections in the animals.

### Cells and nude mice

The cell lines used in this study, KG-1, HL-60, U-937, and THP-1, represent cells arrested at different stages of differentiation along myelomonocytic lineage (reviewed in ref. 34). The relative order of maturation stage of these cells is KG-1, HL-60, U-937, and THP-1 (34). The cells were grown in RPMI 1640 medium (GIBCO; Grand Island, NY) supplemented with 10% FCS and antibiotics. All cell cultures were mycoplasma-free. The nude (immunodeficient) mice used in this study were 3-month-old males of the NIH-1 high fertility strain, routinely bred and maintained under strict pathogen-free conditions at our laboratory as described (35, 36).

### Xenografts and drug treatment

Approximately $2 \times 10^7$ cells were inoculated in each nude mouse, subcutaneously, as described (12, 13). The day of cell inoculation was designated as Day 0. For studies of tumor growth inhibition, drug treatment started on Day 1. In studies of tumor regression, drug treatment started when the tumors reached the size indicated in the text or figure legend. The animals were injected with 9NC as described (12). Briefly, the drug was administered as a fine suspension in Intralipid 20% intramuscularly (i.m.), twice per week, at doses of 4 mg per kg of body weight. Studies in our laboratory have shown that CPT derivatives are much more effective as anticancer agents when administered as suspensions i.m. than i.v. Solubilization of CPT derivatives with aqueous solvents also decreases their antitumor activity when compared with the activity of suspensions (13). Tumor growth and drug toxicity in animals were monitored by measuring the tumor size and the body weight, respectively.

### Preparation of samples for microscopy

Cytospin-prepared slides of untreated and 9NC-treated cells were fixed and stained with Giemsa.

Dried slides with stained cells were covered with immersion oil (Type A, Cargille Laboratories, Cedar Grove, New Jersey) and coverglass. Tumor samples were removed with the aid of a 2-mm biopsy punch (Baker-Cummins Pharmaceuticals, Inc., Miami, Florida), and fixed in 3% formalin solution prior to preparation of histological sections and staining with hematoxylin-eosin at the Pathology Department of St. Joseph Hospital, Houston, Texas. Slides of histological sections and flasks of stained cells were observed under a Zeiss microscope, Axioscope 100, equipped with a Zeiss camera. Photomicrographs were taken on Gold 100 Kodak film.

### Flow cytometry determinations

Cell subpopulations in cultures of untreated and 9NC-treated cells were detected by analysis of the DNA content of the cells using an EPICS-ELITE Laser Flow Cytometer (Coulter Corp.; Hialeah, Florida). Briefly, cells were rinsed in PBS, and suspended at about $10^6$ cells/ml in the staining solution (Coulter), which contained the DNA-binding dye propidium iodide. Incubation in this solution was for 15 min at room temperature. The cells were then subjected to analysis of DNA content. Relative DNA content per cell was measured indirectly by measuring relative fluorescence of propidium iodide which binds stoichiometrically to DNA. However, propidium iodide staining does not distinguish $G_0$ cells from $G_1$ cells, and $G_2$ cells from M cells. A suspension of chicken erythrocyte nuclei (provided by Coulter) was used as reference. Quantitation of DNA content was performed with the aid of the MULTICYCLE program from Phoenix Flow Systems (San Diego, California). The method of cell cycle fitting was based upon the polynomial S-phase algorithm developed by Dean and Jeff (37) with an interactive, nonlinear least squares fit performed by the method of Marquardt (38).

## Results

### Growth inhibition of cells *in vitro*

The effect of the CPT derivatives, 9NC, 9AC, and 12NC, on proliferation of the myeloid leukemia cell lines HL-60, U-937, KG-1, and THP-1 is shown in Fig. 1. Controls included untreated cells and cells treated with PEG alone, that is, the solvent used to prepare the drug suspensions. The final PEG concentration in cell cultures was 0.02%. Apparently, this PEG concentration had no effect on the proliferation of all cell lines tested, since no differences were observed between untreated and PEG-treated cells. Similarly, the inactive CPT derivative 12NC had no effect on cell proliferation even at a concentration of 25 ng/ml. In contrast, 9NC effectively inhibited proliferation of HL-60, U-937, and KG-1 cells, the later cells being more sensitive than HL-60 and U-937. Proliferation of the cells was completely inhibited in cultures treated with 5 ng/ml 9NC for period of time longer than 96 h. However, 5 ng/ml 9NC had little effect on the proliferation of THP-1 cells (Fig. 1), whereas 15 ng/ml 9NC completely inhibited proliferation (not shown). 9NC and 9AC exhibited similar effectiveness on HL-60, U-937, and KG-1 cells, but 5 ng/ml 9AC was more effective on THP-1 cells.

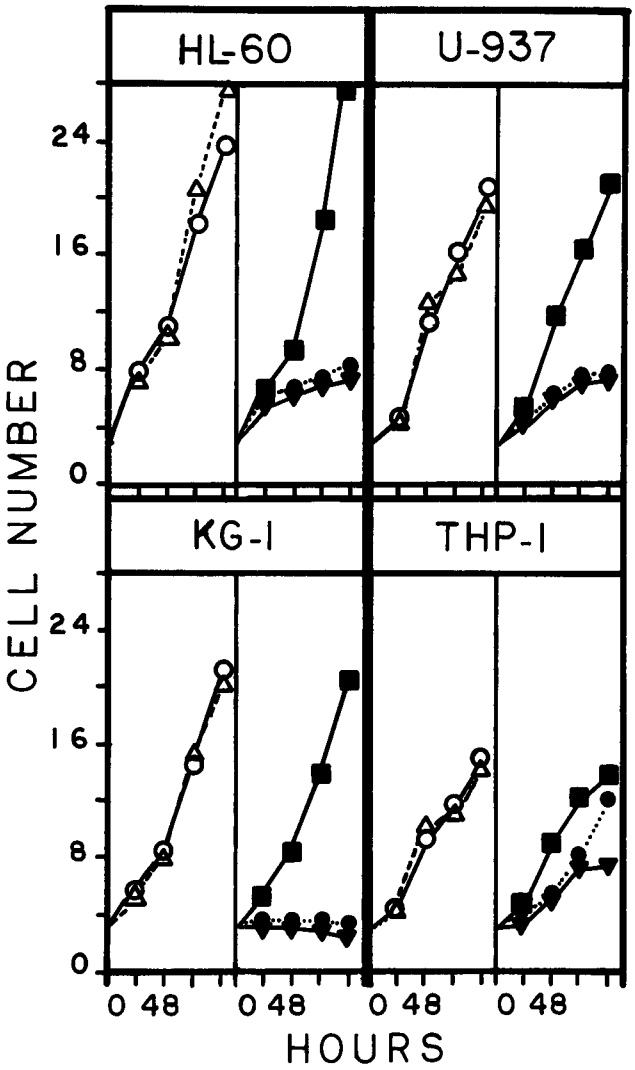


*Fig. 1.* Growth inhibition of leukemia cells *in vitro*. Exponentially grown cultures of myeloid leukemia cells were divided to separate flasks and received various additives. Live cells were counted every 24 h. The cultures received no additive (○); PEG alone (△); and PEG containing 5 ng/ml 9NC (●), 15 ng/ml 12NC (■), and 5 ng/ml 9AC (▼).

### Microscopy of 9NC-treated cells *in vitro*

Observations of inhibition of proliferation of 9NC-treated leukemia cells were accompanied by micro-

scopic observations of these cells stained with Giemsa. Photomicrographs of untreated (panels A, C, E) and 9NC-treated (panels B, D, F) HL-60, U-937, and THP-1 cells are shown in Fig. 2. After 24 h of treatment, a small size increase was observed in several cells of each line, with more enlarged cells observed after 48 h of treatment (not shown) and almost all cells becoming enlarged after 72 h of treatment (B, D, F). The increase was more pronounced in U-937 and THP-1 than in KG-1 and HL-60 cells (Fig. 2; and data not shown). Particularly interesting is the observation that several of the 9NC-treated oversize cells contained more than one nucleus. Also, it can be observed that increases in cell size are accompanied by increases in the size of the nuclei and the amount of nucleolar material.

### Effect of 9NC on cell cycle of leukemia cells *in vitro*

Fig. 3 shows histograms of DNA content of the leukemia cells treated with 9NC for various periods of time. Indirectly, these histograms show percent distribution of cell subpopulations at various phases of the cell cycle. These percent changes in various cell fractions as a function of period of 9NC treatment are shown in Fig. 4. In untreated HL-60 cells, the predominant fractions are in $G_0G_1$ and S, and only a small number of cells in $G_2M$ and $A_0$. Six-hour

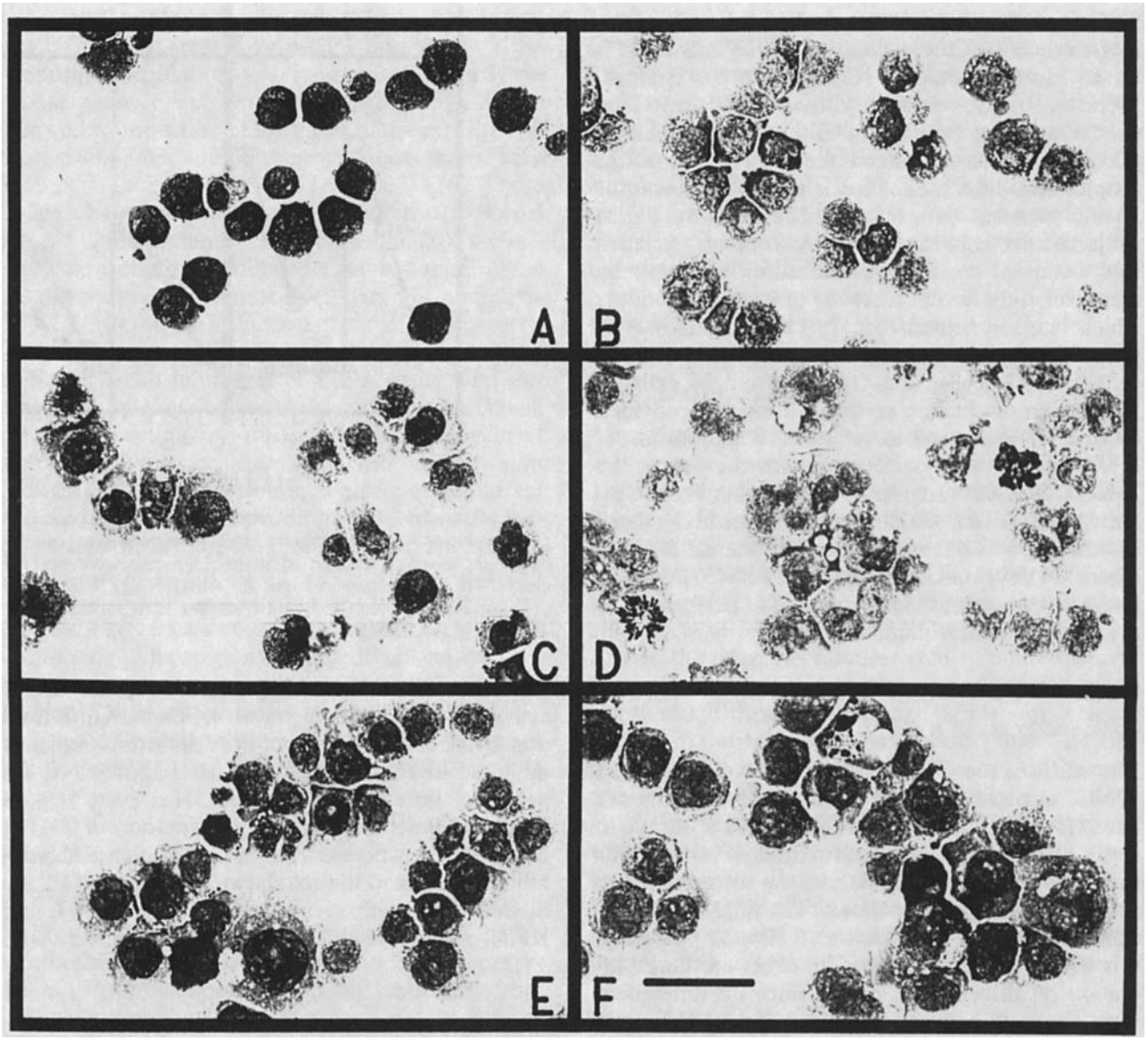


*Fig. 2.* Microscopy of 9NC-treated cells. Untreated and 9NC-treated cells were Cytospin-pelleted on slides, stained with Wright-Giemsa, and photomicrographed at the same magnification. The cells were treated for 72 h with 5 ng/ml 9NC (B, D, F) or PEG alone (A, C, E). The cells shown in this figure are HL-60 (A, B), U-937 (C, D), and THP-1 (E, F). Bar equals 5 μm (panel F).

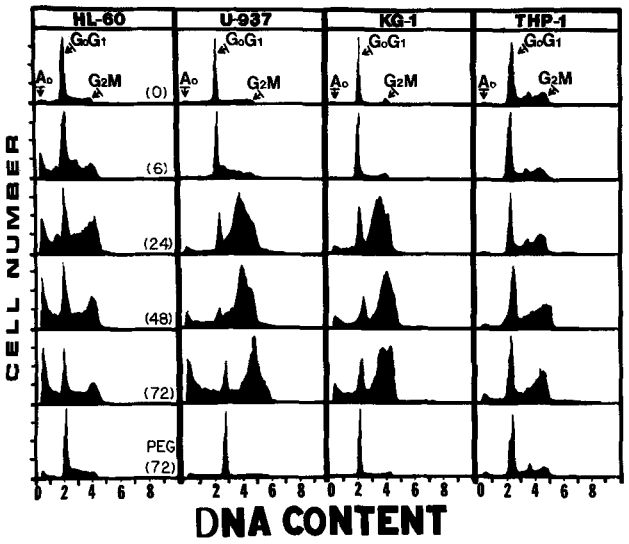


*Fig. 3.* Flow cytometric DNA analysis. Cultures of myeloid leukemia cells received 5 ng/ml 9NC, and cells were removed at 6, 24, 48, and 72 h of treatment for flow cytometric analysis. Control cell cultures received no additive or PEG alone for 72 h. The peaks and regions of the histograms corresponding to cells at various cell-cycle phases are indicated. Hours of treatments are indicated in parentheses.

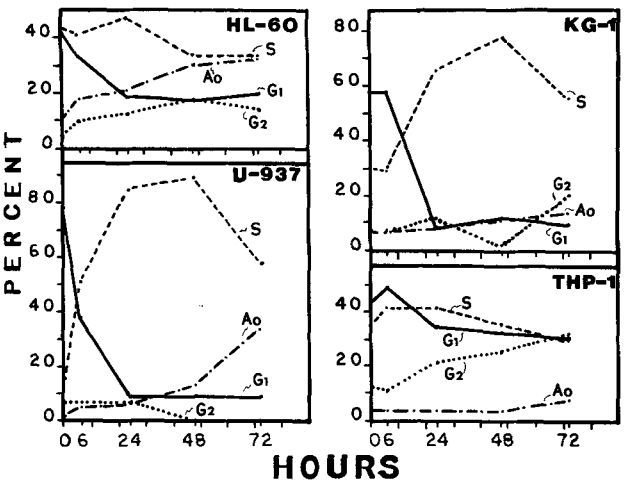


*Fig. 4.* Quantitative effect of 9NC on cell fractions corresponding to specific phases of the cell cycle. Percent of cells at each specific phase of the cell cycle was computed from the histograms shown in Fig. 3. The percent of each phase-specific cell fraction was plotted as a function of period of 9NC treatment of the cell culture. $G_1$, pool of cells in $G_0$ and $G_1$; $G_2$, pool of cells in $G_2$ and M; S, cells in S-phase; and $A_0$, apoptotic cells.

treatment with 9NC increased the cell fraction in $G_2M$ and $A_0$, apparently at the expense of the cell fraction in $G_0G_1$. After a 24-h period of 9NC treatment, the fractions continue to increase in $G_2M$ and $A_0$. Further, there is an increase of the fraction in S-phase. These increases in cell fractions in S, $G_2M$, and $A_0$ take place with a concomitant decrease of the cell fraction at $G_0G_1$. A 48-h 9NC treatment results in a dramatic decrease in the S-fraction, and a smaller decrease in the $G_0G_1$ fraction, while a dramatic increase in the $A_0$ fraction and a small increase in the $G_2M$ fraction take place. No further significant changes in the various fractions of HL-60 cells were observed at 72 h of 9NC treatment (Figs. 3 and 4). PEG alone had no effect on distribution of the fractions along the cell cycle, since no apparent differences were observed between untreated cells and cells treated with PEG alone for 72 h (Fig. 3). Further, KG-1 and U-937 cells exhibit more striking responses than HL-60 to 9NC treatment. A 48-h period of 9NC treatment results in a major fraction at S-phase, whereas the $G_2M$ fraction decreased dramatically. At 72 h of treatment, there were virtually no U-937 cells at $G_2M$, whereas the $G_2M$ fraction increased in KG-1 cells (Fig. 4). Like HL-60 cells, KG-1 and U-937 cells exhibited an increasing $A_0$ fraction as treatment of these cells with 9NC continued. In conclusion, it appears that 9NC initially induces HL-60, KG-1, and U-937 cells to traverse from $G_1$ to S-phase then followed by accumulation of KG-1 cells in $G_2$, or by apoptotic death of HL-60 and U-937 cells. In contrast, histograms of THP-1 cells treated with 9NC show less dramatic changes in their fractions than HL-60, KG-1, and U-937 cells. Interestingly, there was an extensive accumulation of THP-1 cells at $G_2M$ during a 72-h treatment with 9NC (Figs. 3 and 4). It appears that increase in the $G_2M$ fractions results at expense of fractions at $G_0G_1$ and S-phases. It is also interesting that, unlike HL-60, KG-1 and U-937 cells, there was no significant increase in the $A_0$ fraction of THP-1 cells treated with 9NC for 72 h (Fig. 4).

### Treatment of solid tumors induced by leukemia cells xenografted in nude mice

Studies of leukemia cells treated with 9NC *in vitro* were accompanied by studies of leukemia cell-induced tumors in nude mice treated with 9NC. Initially, we tested whether xenografts of the human leukemia cells, HL-60, U-937, KG-1 and THP-1, induce tumors in nude mice. More than 20 nude mice were inoculated with cells of each line. Tumor growth was monitored by daily examination of the animals. No tumors were observed by 30 d postinoculation of HL-60 and THP-1 cells. One-third of the mice inoculated with KG-1 cells developed small tumors 8 to 10 d postinoculation, but all these tumors then regressed spontaneously. However, all 23 nude mice inoculated with U-937 cells developed sizable tumors 5 to 7 d postinoculation. No spontaneous tumor regression was observed in these mice during a 30-d period; instead, the tumor size in-

creased. In one experiment, changes in tumor size and body weight of mice were monitored as a function of time (Fig. 5). It can be seen that the tumor size increased rather rapidly (panel A). Mice with large tumors were sacrificed. All mice with U-937 tumors had to be sacrificed within 40 d postinoculation. It can also be seen that increases in tumor size were accompanied by increases in body weight (panel B). In a parallel experiment, we investigated whether 9NC inhibits the growth of U-937 leukemia tumors in nude mice. For this experiment, 7 mice received xenografts of U-937 cells, and subsequently were treated with 9NC as described in Material and Methods. 9NC-treatment was initiated 1 d after cell inoculation. Measurements of tumor size (panel C) and body weight of mice (panel D) were recorded two or three times a week. The results show that 9NC treatment extensively delayed the growth of U-937 tumors in mice. Measurable tumors developed in 5 mice 26 d after xenografting, 1 mouse developed a tumor 40 d postinoculation (panel C), and 1 mouse remained tumor-free. All mice xenografted with U-937 cells that did not receive 9NC treatment developed measurable tumors in 5 to 7 d (panel A).

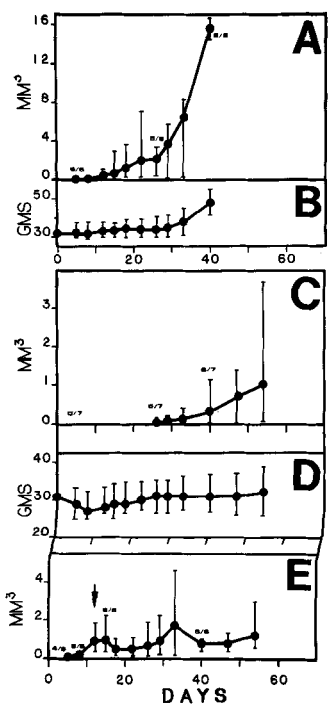


*Fig. 5.* Treatment of U-937 tumors with 9NC. Measurements of average tumor size (A, C, E) and body weight (B, D) of mice. Panels A and B, nude mice xenografted with U-937 cells without receiving 9NC treatment started 1 d after U-937 cell inoculation; and Panel E, 9NC treatment started 12 d after U-937 cell inoculation. Vertical bars indicate highest and lowest measurements. Tumor size was measured in $mm^3$ ( $\times$ 1000). Numerical fractions in each panel indicate number of mice measured (numerator) our of total mice used in the experiment (denominator).

Delay of the growth of U-937 tumors in nude mice was also reflected in the weight of these mice (panel D). There was a 10–15% loss of weight in the initial period of 9NC treatment, but this was followed by a weight recovery and subsequently a slight increase as the tumors grew.

Since 9NC induces complete regression of human malignant melanoma tumors established in nude mice (15), we investigated whether 9NC can induce regression of U-937 tumors in nude mice. For this study, 6 animals were inoculated with U-937 cells and tumor growth was monitored (panel E). Four mice had measurable tumors 5 d postinoculation, whereas 1 mouse developed a tumor in 8 d. 9NC treatment of all 6 mice started 12 d postinoculation and continued twice a week. The 6th mouse developed a measurable tumor 15 d postinoculation, that is, 3 d after initiation of 9NC treatment. The tumors initially regressed in the treated mice, but growth of these tumors resumed after a while (panel E). One mouse died before measurements were taken on Day 40, and this led to a decrease in calculations of the average tumor size. The remaining 5 mice had tumors that continued to grow while these mice were receiving 9NC treatments.

### Tumor histology

Changes in tumor size in 9NC-treated animals were also accompanied by observations of biopsy samples removed from these tumors. Stained preparations of tumor sections are shown in Fig. 6. Each section was photographed at low (left panels) and high (right panels) magnification to show the histology and the cytology of the tumor. Untreated U-937 tumors exhibit mostly myeloid cells which are pleiomorphic with relatively little cytoplasm. The nuclei have a variety of shapes and contain nucleoli that stain heavily (panels A). One treatment with 9NC resulted in cytological changes of the tumor, including enlargement of the leukemic cells, the nuclei, and nucleolar material (panels B). Also, there is more spacing among the enlarged cells. After four treatments, there was even more spacing around each cell, decreased number of leukemic cells, and cells with disrupted cytoplasm (panels C). Also present are several macrophages that presumably infiltrated the tumor. After seven 9NC treatments, the tumor included more leukemic cells, and less macrophages (panels D). Finally, the tumor contained many leukemic cells after ten 9NC treatments (panels E) and resembled the untreated tumor (panel A). The morphological changes in the U-937 tumor (Fig. 6) support the observations on tumor size (Fig. 5), that is, 9NC treatment initially results in a partial regression of the tumor, but afterwards growth is resumed despite continuation of the treatment.

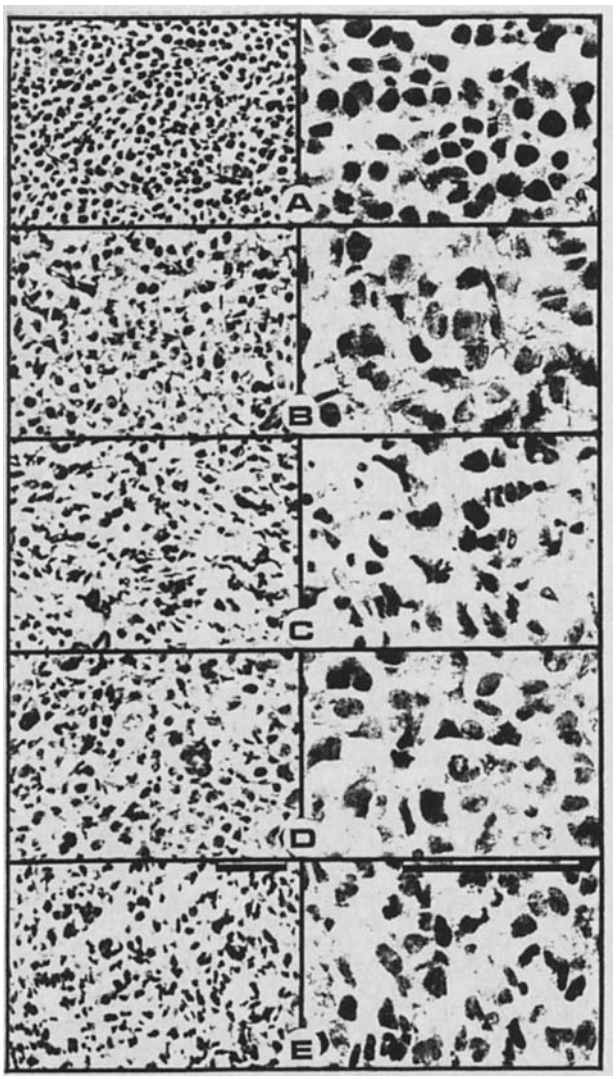


*Fig. 6.* Microscopy of histological sections of U-937 tumor. Panel A, untreated tumor; Panel B, 1 d after the first 9NC treatment; Panel C, 1 d after the fourth treatment; Panel D, 1 d after the seventh treatment; and Panel E, 1 d after the tenth treatment. Bar equals 50 μm.

## Discussion

In this study, we have shown that treatment of human leukemia cell lines HL-60, KG-1, U-937 and THP-1 with CPT derivatives *in vitro* results in growth inhibition and enlargement of these cells and their nuclei. The cell lines HL-60, KG-1, U-937, and THP-1 represent cells arrested at different stages of differentiation along myelomonocytic lineage (34) and have been used previously by other investigators in CPT studies (25, 26, 28, 30). In general, concentrations of 9NC and 9AC that completely inhibited growth of HL-60, KG-1 and U-937 cells *in vitro* were not adequate to inhibit completely growth of THP-1 cells that require drug concentrations of 25 ng/ml or higher. The reason that 9NC and 9AC exert lower antiproliferative activity on THP-1 cells is not yet known. One possibility is that THP-1 cells are less sensitive to the drugs because they are more mature than the other leukemia cells. However, this correlation has not been proven yet.

The 9NC and 9AC concentrations used in this study are lower than the CPT concentrations reported to inhibit growth of HL-60, KG-1, and U-937 cells *in vitro* (24, 25, 27–30). Predictably, high drug concentrations result in rapid growth inhibition of these cells and, therefore, early events involved in the response of the cells to CPT derivatives may be overlooked. For example, we have shown that treatment of U-937 cells with very low concentrations of CPT derivatives (0.5 to 5 ng/ml) induces a temporal overexpression of mRNAs transcribed by the early response genes c-*jun*, *jun*-B, and c-*fos* (25), and this mRNA overexpression has been associated with programmed cell death or apoptosis (25, 26). It has also been reported that low concentrations (20 ng/ml) of CPT arrest human myeloid U-937, HL-60, and KG-1 cells in S- and $G_2$-phases of the cell cycle (26, 28–31), while higher CPT concentrations induce death by apoptosis of cells traversing these phases (31). In agreement with these findings, we also observed that 9NC induced accumulation of the leukemic cells in S and $G_2$, and further showed that this effect on the cell cycle of the drug-treated leukemia cells was followed by increasing numbers of apoptotic HL-60 and U-937 cells. In contrast, only a small number of apoptotic cells was present in drug-treated cultures of KG-1 and THP-1 cells. Apparently, these findings do not correlate with the stage of cell maturation or the length of doubling time of the cells. One possibility is that 9NC-induced arrest of cells in $G_2$ or death by apoptosis depends on the presence of certain cellular factors. For example, it has been reported recently that there is a positive correlation between levels of MYC protein in normal fibroblasts and induction of programmed death in these cells (39). We have not investigated yet whether there is a correlation between levels of MYC protein and/or other cellular factors in the leukemic cells and the differentiation response of these cells to 9NC treatment.

The observed enlargement of the 9NC-treated leukemic cells has also been observed in human malignant melanoma, breast, and ovarian carcinoma cells treated with CPT derivatives, whereas no size increase has been observed in normal human and mouse cells treated with these drugs *in vitro* and *in vivo* (15; and unpublished data). The fact that CPT derivatives have similar effects on tumor-derived and immature hematopoietic cells indicates that these drugs interfere with the cell growth process at a key stage, resulting in growth imbalance of tumor and immature cells. Further, previous studies in our laboratory have shown that 9NC and other CPT de-

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