ABSTRACT

In a recent study, we showed that the plant alkaloid camptothecin (CPT) and its derivatives 9-nitro-CPT (9NC) and 9-amino-CPT (9AC) inhibit growth of both human melanocytes (MEL cells) and their malignant counterparts, malignant melanoma (BRO) cells in vitro. This growth inhibition was accompanied by an increase in the size of BRO cells followed by death, whereas cell size increase and death were not observed in MEL cells. In this study, we have extended those investigations to identify parameters and factors that can modulate the cytotoxic action of 9NC against BRO cells in culture. MEL cells treated with 9NC accumulate at the S/G2 boundary of the cell cycle and remain there for a prolonged period of time with only a small number of cells dying by apoptosis. The extent of accumulation correlates with the length of 9NC treatment and/or 9NC concentration in the cell culture. Furthermore, treatment with low 9NC concentrations for a prolonged time or treatment with high drug concentrations results in a fraction of MEL cells with hyperdiploidy. In contrast, 9NC-treated BRO cells are arrested in the S phase before they die by apoptosis. Interestingly, lower 9NC concentrations are more effective than higher concentrations in inducing apoptosis. Once 9NC initiates the process of apoptosis in BRO cells, these cells are irrevocably committed to it and continue to die even after removal of the drug from the culture. The drug effectiveness to induce apoptosis correlates with the stage of the S phase, in which it affects DNA replication, with late stages resulting in higher numbers of apoptotic cells. Finally, although various 9NC concentrations result in inhibition of BRO cell proliferation, higher 9NC concentrations produce more enlarged BRO cells as assessed by microscopy. Taken together, these observations provide useful information for clinical application of 9NC as a chemotherapeutic agent against malignant melanoma.

INTRODUCTION

The plant-derived CPT3 is an alkaloid that first demonstrated high antiproliferative and toxic activity against the murine leukemia L1210 (1). Subsequently, it was shown that CPT was cytotoxic in vitro and in vivo for a large number of human malignant cells (2–12; reviewed in Refs. 13 and 14). Treatment of cultured human malignant melanoma cells with CPT and its derivatives 9NC and 9AC results in increased cellular, nuclear, and nucleolar size with frequent abnormal divisions of the nucleus (8). Size increase is preceded or accompanied by cessation of cell growth (8). Similar events were observed in cells of human melanoma tumors growing as xenografts in nude mice and treated with CPT, 9NC, and 9AC (8).

We have recently shown that exposure of U937 leukemia cells to CPT, 9NC, and 9AC results in an 80- to 100-fold increase in expression of c-jun and jun-B mRNAs followed by degradation of cellular DNA characteristic of cells undergoing death by apoptosis (7). In earlier flow cytometry studies, it was shown that CPT-treated human leukemia cells exhibit DNA degradation in S phase or are arrested in S and G2 phases, depending on whether the leukemia cells are of myeloid or lymphoid lineage (4, 5). A recent report has shown that the drug-treated leukemia cells are preferentially arrested at the G2/M phase of the cell cycle with an apparent induction of differentiation markers in U937 and other human myeloid cells including HL-60 and THP-1 (15). Also, recently we showed that 9NC-treated HL-60 and U937 cells die by apoptosis while traversing from S toward G2 phase of the cell cycle, while 9NC-treated KG-1 and THP-1 cells accumulate in G2 phase but resist death by apoptosis (12). The rationale for the differential activity of CPT and its derivatives on the leukemia cells has not yet been reported. In other studies, we have shown that 9NC elicits differential responses in the cell cycle of nontumorigenic and tumorigenic human cells in vitro (10, 11). Specifically, in the presence of 9NC, breast and ovarian cells that do not induce tumors in nude mice accumulate at the G2 phase, whereas cells that induce tumors in nude mice die by apoptosis when they traverse the S phase (10, 11). Unlike nontumorigenic and tumorigenic cell lines, CPT-treated normal human melanocytes and fibroblasts do not increase in size, although their growth is halted in vitro (8).

It has been demonstrated that CPT exerts its action on cells while they are actively involved in DNA synthesis (16). Specifically, CPT interferes with the breakage-reunion process of the nuclear enzyme topoisomerase I by stabilizing the covalent adducts between DNA and enzyme termed “cleavable complexes,” thus interfering with the process of DNA breakage-reunion (reviewed in Ref. 17). This event results in various effects including inhibition of DNA replication (18), altered regulation of p34<sup>cdc2</sup>/cyclin B (19), termination of RNA transcription at sites of complex formation (20), induction of expression of early-response genes (7, 21), induction of differentiation (15; 22, 23), and ultimately, internucleosomal DNA fragmentation (7, 15), a characteristic of programmed cell death or apoptosis (reviewed in Refs. 24 and 25). The sensitivity of malignant cells to CPT and its derivatives has been correlated positively with topoisomerase I activity and/or drug-induced accumulation of cleavable complexes (13, 14, 17). However, the current understanding of the CPT-induced cytostatic or cytotoxic effects remains incomplete. For example, it is unclear how CPT and its derivatives are cytostatic for human nontumorigenic cells, while they are cytotoxic for tumorigenic cells (8, 10, 11, and this study).

In this study, we have used the CPT-derivative 9NC to investigate its toxicity and effectiveness on human malignant melanoma cells and their normal counterparts, the melanocytes. The results show that using the same low 9NC concentration, the drug is cytostatic for normal melanocytes but cytotoxic for malignant melanoma cells. Furthermore, we show that the extent of 9NC-induced toxicity in malignant melanoma cells correlates with the drug concentration and period of treatment.

MATERIALS AND METHODS

Drugs. CPT and its derivative 9NC were prepared and purified in our laboratory according to published procedures (26). The drugs, as fine suspensions in polyethylene glycol (PEG 400; Aldrich, Milwaukee, WI), were divided into small aliquots and stored at -70°C until used.

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3 The abbreviations used are: CPT, camptothecin; 9NC, 9-nitrocamptothecin; 9AC, 9-amino camptothecin; PEG, polyethylene glycol; MEL, melanocytes.

4 Unpublished data.
Cells. BRO melanoma cells were derived from a biopsy of a human primary melanoma of the skin and then transplanted in nude mice, where they induce highly invasive tumors without a characteristic pattern of metastasis (27). The cells grow in RPMI 1640 supplemented with 10% fetal calf serum and penicillin-streptomycin at 37°C in a humidified CO2 incubator. The normal counterparts of BRO melanoma cells, human epidermal MEL, were obtained from Clonetics Corporation (San Diego, CA), grown in melanocyte growth medium, and used at passages 4–8. BRO and MEL cell cultures were treated with various 9NC concentrations indicated in the appropriate text section or figure. Untreated and 9NC-treated cells were stained for microscopy or removed by trypsinization and counted or subjected to flow cytometry studies to determine DNA content.

Microscopy and Flow Cytometry. Cells attached on plastic Petri dishes were stained with methylene blue and then observed under a Zeiss Axioscope microscope equipped with an electronically self-adjusted camera. Microphotographs were taken on Kodak 200/plus film. DNA content of cells was determined by flow cytometry using an EPICS-ELITE laser flow cytometer (Coulter Corp., Hialeah, FL) and analyzed with the aid of the MULTICYCLE program from Phoenix Flow Systems (San Diego, CA) as described (10, 11).

We have used this methodology to analyze and quantitate changes in fractions of various human cells treated with CPT derivatives in vitro (10–12). Also, this methodology reveals the fraction of the total cell population that consists of cells with DNA content less than the DNA content of cells at G0+G1 (10–12). Use of flow cytometry and specific fluorescent dyes has assessed that the cells of this fraction have entered programmed cell death or apoptosis and differ from cells dying by necrosis in several features including: (a) apoptotic cells exhibit a decreased stainability with DNA-specific fluorochromes because of specific endonuclease-generated DNA-fragments and their subsequent loss from the cells; (b) apoptotic cells, but not necrotic cells, preserve the integrity of the plasma membrane, the mitochondrial transmembrane potential, and the adenosine triphosphate-dependent lysosomal proton pump; and, (c) endonuclease and proteinlysis are coupled in apoptotic cells, whereas necrotic cells exhibit high proteolytic but low or no nucleolytic activities (reviewed in Ref. 28).

RESULTS

Growth and Morphology of Cells Treated with 9NC. For these studies, MEL and BRO cells were seeded in T25 flasks in their respective culture media 24 h prior to addition of 9NC. Final 9NC concentrations in cell cultures were 20, 40, and 80 nM. Control cells received PEG alone. Cells were counted every 24 h after 9NC addition. Fig. 1 shows the number of MEL and BRO cells counted for 72 h. Exponentially growing control MEL and BRO cells have estimated doubling times of 44 h and 20 h, respectively. All three 9NC concentrations tested effectively inhibited growth of the cell cultures.

In addition to monitoring cell proliferation, cells were stained and examined by microscopy. Fig. 2 is a composite of microphotographs of BRO cells treated with 20 and 80 nM 9NC. Control cell cultures received no additive (Fig. 2A) or PEG alone (Fig. 2E). Continuous treatment of BRO cells with 9NC results in an increased size of the cells. Eventually, many multinucleated cells are present in cultures treated with 20 nM 9NC for 6 days (Fig. 2D). Furthermore, cells treated with 80 nM 9NC (Fig. 2, F–H) increase in sizes larger than the sizes of cells treated with 20 nM 9NC (Fig. 2, B–D) for the same period of time. However, unlike 20 nM-treated cultures, 80 nM-treated cultures contain no or a small number of multinucleated cells after 6 days of treatment (Fig. 2H). It should be noted that the cells remained unchanged in the control cultures (Fig. 2, A and E). Finally, neither 9NC concentration affected the size of MEL cells in agreement with a previous report (8).

Changes in Cell Cycle Depend on the Length of Treatment Period. In this study, we monitored changes in the cell cycle of MEL and BRO cells during their treatment with 9NC for 120 h. Control cell cultures received the drug vehicle alone that is PEG, whereas treated cells received 20 nM 9NC in PEG. At various periods of 9NC-treatment, the relative DNA content of the cells was measured by flow cytometry. The results (histograms) of this study are shown in Fig. 3. The majority of untreated MEL cells is in G1 phase with a small number of cells in G2 (Fig. 3A). Also, a small number of cells are in S phase (histogram area between G1 and G2 peaks) in agreement with the observation that MEL cells have a long doubling time (Fig. 1). Finally, a very small fraction of the cultured cells (Fig. 3A, peak AP) has a DNA content less than the DNA content of diploid cells (Fig. 3A, peak G1). These AP cells die by apoptosis (28). No significant changes are observed in the cell cycle of MEL cells treated with 9NC for 6 h (Fig. 3B). However, 9NC treatment for 24 h results in an arrest of a large fraction of cells in G2 (Fig. 3C). The increase of cells in the G2 fraction continues as 9NC treatment continues for 120 h and eventually most of the MEL cells accumulate in G2 (Fig. 3, D–G). During this transition of cells from G1 to G2 phase, only a small number of cells was found in the AP peak. Treatment of MEL cells with PEG alone for 120 h does not result in significant changes in the cell cycle (Fig. 3H), indicating that 9NC alone is the causative agent for the changes in the fractions of MEL cells.

In parallel experiments, we monitored changes in the fractions of BRO cells exposed to 9NC for 120 h (Fig. 3, J–O). Control cultures received no additive (Fig. 3J) or PEG alone (Fig. 3O). Like MEL cells, the majority of the BRO cells are in G1 in the untreated culture (Fig. 3J). However, unlike MEL, BRO cells include large fractions in the S and G2 phases, indicating that these cells divide actively. Almost all BRO cells are in middle S phase following a 24-h treatment with 9NC (Fig. 3K). Longer 9NC treatment results in further shift of cells in late S phase with some cells being arrested in G2 (Fig. 3, L–O). As the 9NC treatment becomes longer, there is an increase in the fraction of apoptotic cells with a concomitant decrease in the S and G2 fractions. No significant changes take place in the fractions of BRO cells treated with PEG alone for 120 h (Fig. 3P).

Percentage changes in fractions of 9NC-treated MEL and BRO cells are shown in Fig. 4. It can be seen that the G0+G1 fraction of the MEL cells decreases dramatically at 48 h of 9NC treatment and remains at about 20% for a prolonged period of treatment. It is also apparent that this decrease in the percentage of the G0+G1 fraction is due to the increase of percentage in the fraction primarily at G0+M and less at S with small percentage changes in the AP fraction. In
Fig. 2. Microscopy of human melanoma cells treated with 9NC. Cell cultures received 9NC to final concentrations of 20 nM (B, C, and D) and 80 nM (F, G, and H) and were photomicrographed at 24 h (B and F), 96 h (C and G), and 144 h (D and H) of treatment. Control cells received no additive (A) or were exposed to PEG alone for 144 h (E).

Contrast, a 24-h treatment of BRO cells with 9NC results in a virtual elimination of the G0+G1 fraction and a decrease in the percentage of S fraction with a concomitant dramatic increase in the percentage of the AP fraction and a smaller increase in the percentage of G2+M fraction. However, this percentage of G2+M fraction decreases after prolonged treatments and only apoptotic cells remain (see below). It should be noted that computer calculations of the percentage of cell fractions, shown in Fig. 2, are estimates derived from the DNA histograms shown in Fig. 1. However, these estimates are not accurate because the 9NC-induced perturbations in the cell cycle result in DNA histograms that are not discretely separated. Difficulties in accurate numerical analysis of DNA fraction data have been reviewed extensively in a recent publication (29).

Cell Cycle Arrest of MEL Cells Depends on 9NC Concentration. Treatment of MEL cell cultures for 24 h with increasing 9NC concentrations results in accumulation of MEL cells in late S phase. This is evident when the G1 peaks of the flow cytometry histograms are aligned (Fig. 5, A-D). The number of cells arrested in late S/G2 correlates positively with the drug concentration in the culture, regardless of the period of treatment. In other words, the higher the drug concentration, the larger the fraction of the arrested cells. However, it is apparent that prolonged treatments with higher drug concentrations result in more dramatic increases in the arrested fraction, which eventually includes the majority of the cells. Also, a small number of hyperdiploid MEL cells is present in cultures that received prolonged treatments with increased drug concentrations. The hyperdiploid cells generate the histogram area indicated by arrows in Fig. 5. Finally, 9NC-induced quantitative changes in the cell fractions are accompanied by no or little generation of apoptotic cells.

9NC-induced Toxicity in BRO Cells Depends on Drug Concentration. In this section, we investigated whether use of various 9NC concentrations modulate the sequence and/or timing of specific events associated with toxicity of 9NC against BRO melanoma cells. For these studies, the cells were treated with three different drug concentrations, 20, 40, and 80 nM, followed by DNA content analysis every 24 h using flow cytometry. The histograms of these studies are shown in Fig. 6. Treatment of the cells for 24 h with 9NC resulted in arrest of the cells in S phase (Fig. 6, B-D) and a concomitant absence of cells in G1, the major cell fraction in the untreated cultures (Fig. 6A). Interestingly, as the 9NC concentration increases, the cells are arrested in the S phase region closer to the G1/S boundary. This can be monitored by the fact that the histogram peak of the arrested cells (Fig. 6, B-D) moves closer to the G1 peak of the untreated cells (Fig. 6A). Similar patterns are observed in cells treated with increasing drug concentrations for 48 h (Fig. 6, F-H). In addition, 48-h treated cells contain a small number of apoptotic cells (AP peak of histograms).
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increases, the BRO cells are arrested in earlier stages of the S phase. These results are in agreement with the results obtained when drug treatment was applied for 24 h and 48 h as discussed above. Taken together, these results indicate that there is a correlation between 9NC-induced cell killing and stage of S phase in which the cells are arrested.

Is the Effect of 9NC on Cells Reversible? This question stemmed from the possibility that upon removal of 9NC from the cell culture, the cells will resume their growth. To investigate this, MEL and BRO cells were exposed to 20 nM 9NC for various periods; then the cells were transferred in 9NC-free medium. After 48 h in 9NC-free medium, the cells were analyzed for DNA content by flow cytometry. Control cell cultures did not receive 9NC at all or remained under 9NC treatment for the duration of the experiment. The results are shown in Fig. 7. Continuous treatment of MEL cell cultures with 9NC resulted in accumulation of most cells at G2, a reduced fraction at G1, and appearance of hyperdiploid cells (Fig. 7, A, B, D, E, G, and H) as discussed above (Fig. 6). Transfer of 9NC-treated MEL cells to 9NC-free medium after 48-h treatment does not significantly alter the pattern of DNA content histograms, that is, most of the cells remain

Fig. 3. Flow cytometry of cells treated with 9NC. MEL and BRO cells were exposed to 20 nM 9NC, and changes in relative DNA content were monitored as a function of period of treatment. MEL cells, A-H; BRO cells, I-P. The cells received no treatment (A and I) or were treated with 9NC for 6 h (B and J), 24 h (C and K), 48 h (D and L), 72 h (E and M), 96 h (F and N), and 120 h (G and O). Additional control included cells exposed to PEG alone for 120 h (H and P). AP, apoptotic cells; G1, G0 + G1 cells; and G2, G2 + M cells.

Fig. 4. Percentage distribution of cell fractions in cultures treated with 9NC. The percentage of cells in each fraction was calculated from the histograms of flow cytometry shown in Fig. 3.

Furthermore, the relative number of AP cells increases dramatically in cultures treated for 72 h with 20 nM 9NC (Fig. 6J), whereas a smaller AP cell fraction is present in cultures treated for 72 h with 80 nM 9NC (Fig. 6L). A 72-h treatment with the intermediate 9NC concentration of 40 nM (Fig. 6K) results in an AP cell fraction that sizewise is between the AP fractions shown in Fig. 6, J and L. Further analysis of these histograms (Fig. 6, J-L) shows that, as the 9NC concentration

Fig. 5. Relative DNA content of fractions in MEL cell cultures treated with 9NC. Cultures of MEL cells were treated with PEG alone (A, E, and J) or PEG and 20 nM (B, F, and J), 40 nM (C, G, and K), and 80 nM (D, H, and L) 9NC. The cells were exposed to additives for 24 h (A-D), 48 h (E-H), and 72 h (I-L).

Fig. 6. Relative DNA content of fractions in BRO cell cultures treated with 9NC. Cultures of BRO cells were treated as the cultures of MEL cells (see Fig. 5).
In this report, we have demonstrated that the CPT derivative 9NC arrests normal human melanocytes at the S/G2 boundary, while malignant melanoma cells are first arrested at the S phase and then die by apoptosis. The effects of 9NC on the cell cycle of normal and malignant melanocytes were monitored by flow cytometry. Previous flow cytometry studies have shown that CPT and its derivatives, 9NC and 9AC, halt proliferation of cells in vitro as a result of cell arrest at the S and G2 phases of the cell cycle (4, 5, 10–12, 15). Furthermore, it has been shown that S phase arrest is a prerequisite for the drug-induced toxicity that ultimately results in programmed cell death or apoptosis (19). However, this mechanism of CPT action may exist in malignant but not in nonmalignant cells. We have recently reported that CPT derivatives are cytostatic for nontumorogenic cells but cytotoxic for tumorogenic cells (10, 11). Both nontumorogenic and tumorogenic cells used in those studies derived from biopsies of human tumors but differed in their ability to induce tumors in nude mice (10, 11). Specifically, CPT derivatives cause arrest of nontumorogenic cells in G2, whereas drug-treated tumorogenic cells that enter S phase die by apoptosis (10, 11). Other reports have also described arrest of CPT-treated cells in S and G2 phases (4, 5, 15), but no correlations were made with the tumorogenic abilities of the cells in animals. In the present report, we show that drug-treated normal MEL cells accumulate at the S/G2 boundary, and the extent of cell accumulation correlates positively with the duration of drug-treatment and the concentration of drug used. Interestingly, under experimental conditions that favor accumulation of most drug-treated MEL cells at S/G2, there is appearance of a substantial number of hyperdiploid cells. A similar appearance of hyperdiploid cells has been reported for human leukemia and nontumorogenic breast cells that are arrested at S/G2 in the presence of CPT derivatives (5, 10). Furthermore, removal of the drug from the environment of MEL cells does not result in reentry of these cells in the cycle. Similar responses have been observed in a variety of other normal human cells treated with 9NC prior to transferring these cells to 9NC-free medium.4 This contrasts with some recent studies in which nontumorogenic breast and ovarian cells are arrested at S/G2 in the presence of 9NC, but removal of the drug from the cultures allows the cells to reenter the cell cycle (10).4 Taken together, these results raise the possibility that reentry in the cell cycle depends on the ability of the untreated, cultured cells to propagate only for a small number of passages or for many.

Another interesting observation is that only a small portion of MEL cells appear to die by apoptosis in the continuous presence of or following treatment with 9NC (10, 11). We have reported similar observations for nontumorogenic breast and ovarian cells treated with 9NC (10, 11). Also, of interest is the observation that 9NC results in enlargement of nontumorogenic cells derived from tumor biopsies, whereas the same drug does not induce enlargement of MEL cells and fibroblasts (8).4 It should be noted that the ability of CPT derivatives to induce enlargement of some cells but not of others does not correlate with the antiproliferative activity of these drugs. Because of these observations, we suggest that CPT derivatives have different actions on cells with balanced and unbalanced growth.

In contrast to 9NC-treated MEL cells that are arrested at S/G2, 9NC-treated malignant BRO melanoma cells die by apoptosis. As assessed by flow cytometry studies, 9NC-treated BRO cells traversing the S phase do not reach G2, but they directly enter the apoptosis peak. However, it appears that the drug concentration plays an important role on how far in the S phase the BRO cells will advance. For example, at low drug concentrations, the cells advance to late stages of the S phase, but as the drug concentration increases, the cells advance less in the S phase, i.e., they accumulate closer to the G1/S boundary (Fig. 6). Remarkably, the percentage of apoptotic cells increases as their arrest takes place in later rather than earlier stages of the S phase. Therefore, although paradoxical, it appears that low drug concentrations are more effective than high drug concentrations in the ability of inducing apoptosis in BRO cells. In general, there is good evidence that CPT exerts its killing activity against malignant cells in S phase (16). Furthermore, it appears that low 9NC concentrations allow more cells to complete DNA replication; therefore, more topoisomerase I-DNA complexes are available as targets for 9NC. However, a quantitative correlation of induction of apoptosis with early and late S phase has not been made yet. Nevertheless, this observation may be useful from a clinical viewpoint. Furthermore, the long term effectiveness (Fig. 3) of low drug concentration on BRO cells remains unaltered even when the drug is removed (Fig. 7), indicating that once the drug-treated cells enter the process of apoptosis, they are irreversibly committed and the continuous presence of the drug is not required. This observation may also have clinical importance from the viewpoint of the timing of drug administration. Moreover, lower drug concentrations result in less cell enlargement than higher drug concentrations (Fig. 2). This observation should be taken into consideration when a tumor, to be treated with a CPT-derivative, is located in or near a vital organ of the body, e.g., lung or brain, where a rapid increase in tumor size may be life threatening.

Although topoisomerase I has been implicated in the cytotoxic action of CPT and its derivatives, it remains unclear how these agents (a) are cytotoxic for human tumorogenic cells but cytostatic for non-

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**Fig. 7. Changes in fractions of cells transferred from 9NC-containing to 9NC-free medium. A-I, MEL cells; J-R, BRO cells; A,J, + PEG alone for 48 h; B,K + 9NC/PEG for 48 h; C,L, + 9NC/PEG for 48 h and then transferred to 9NC-free medium; D,M, + PEG for 96 h; E,N, + 9NC/PEG for 96 h; F,O, + 9NC/PEG for 96 h and then transferred to 9NC-free medium for 48 h; G,P, + PEG for 144 h; H,Q, + 9NC/PEG for 144 h and then transferred to 9NC-free medium for 48 h.**
tumorigenic cells (8, 10, 11, and this study); (b) interfere with the regulatory mechanisms(s) of balanced cell growth (8, 10, 11); (c) induce differentiation of certain cell types (22, 23); and (d) are more effective in induction of apoptosis when they interfere with DNA replication at late rather than early stages of the S phase (this study).

It is apparent that, in addition to topoiso merase I, other cellular components and/or factors regulate the various activities of camptothecins. Recent reports have shown that the mechanism of apoptosis in human cells may be regulated by protein activation/modification such as phosphorylation/dephosphorylation (30—31) and expression of various protooncogenes, including c-Jun (7), c-fos (32), c-myc (33—36), bcl-2 (34—39), c-ras (40), Apo-1 (41), and p53 (42, 43). Furthermore, the process of apoptosis in various cell types seems to require interaction of two or more oncopgenes (44, 45).

However, the present and other studies (10, 11) have indicated that, regardless of the factors involved in the process of apoptosis, CPT and its derivatives can selectively trigger this process in malignant but not in nonmalignant cells. To our knowledge, this remarkable finding has not been described for any anticancer drug other than camptothecins.

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Cytotoxic Efficacy of 9-Nitrocamptothecin in the Treatment of Human Malignant Melanoma Cells *in Vitro*

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