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Development of resistance to 9-nitro-camptothecin by human leukemia U-937 cells *in vitro* correlates with altered sensitivities to several anticancer drugs

Panayotis Pantazis, John Mendoza, Albert DeJesus, Janet Early, Marcus Shaw and Beppino C Giovanella

The Stehlin Foundation for Cancer Research, 1918 Chenevert Street, Houston, TX 77003, USA.
Tel: (+1) 713 756 5750; Fax: (+1) 713 756 5783.

We have recently reported that exposure of human leukemia U-937 cells to progressively increasing concentrations of 9-nitro-camptothecin (9NC) resulted in cell sublines exhibiting various levels of resistance to 9NC. Here, we report responses of wild-type (U-937/wt) and 9NC-resistant (U-937/CR) cells to various anticancer drugs used extensively in cancer chemotherapy. U-937/CR cells were more sensitive than U-937/wt cells to several commonly used drugs of diverse origin including the topoisomerase II-directed drugs amsacrine, etoposide and daunorubicin; the vinca alkaloid vincristine; and the antimetabolite methotrexate. No responses were induced by carmustine in either cell type, whereas similar responses were induced by cytarabine. The sensitivity to the drugs was investigated by monitoring cell proliferation, by determining cell cycle perturbations assessed by flow cytometry analysis of DNA content and by microscopy of stained cells. The results in this report indicate that development of 9NC resistance by the U-937 cells is accompanied by increased sensitivities to other anticancer drugs *in vitro* and very likely *in vivo*.

Key words: Camptothecin, flow cytometry, leukemia cells, resistance.

Introduction

We have recently shown that the plant-derived alkaloid agent camptothecin (CPT) and its semisynthetic, water-insoluble derivatives 9-nitro-CPT (9NC) and 9-amino-CPT (9AC) elicit differential responses in the cell cycle of human cells *in vitro* that correlate with the ability of these cells to induce tumors when xenografted in nude mice.[13] Specifically, human breast and ovarian cells and melanocytes that do not induce tumors in nude mice accumulate at the boundary of late-S/G_2 phase in presence of 9NC, whereas the tumorigenic counterparts of the cells mentioned above, die by apoptosis while they traverse the S phase in the presence of 9NC.[13] CPT exerts its action through the nuclear enzyme topoisomerase I (topo I) by stabilizing the covalent adducts between topo I and DNA, termed 'cleavable complexes', and thus interfering with the mechanism of DNA breakage-reunion (reviewed elsewhere[46]). Further, the sensitivity of malignant cells to CPT and its derivatives has been correlated positively with topo I activity and/or drug-induced accumulation of cleavable complexes.[46] However, the current understanding of the CPT-induced cytostatic or cytotoxic effects remains incomplete.

In other studies, resistance of murine, hamster and human cells to CPT has been correlated with decreased expression or alterations, i.e. point mutations, in the topo I gene of the resistant cells[7–12] Within this context, we have shown that development of 9NC resistance by human leukemia U-937 cells *in vitro* initially correlates with the appearance of a point mutation in the topo I gene that results in an enzyme with increased resistance to 9NC,[12] while further cell resistance to 9NC correlates with a decrease in the synthesis of topo II.[13]

In this study, we have examined responses of 9NC-resistant U-937 cells to topo II-directed and other drugs, and show that the resistant cells have altered their sensitivities to some of these drugs. These observations may have clinical implications.

Materials and methods

Drugs

CPT purification and subsequent semisynthesis of 9NC were conducted in our laboratory according to

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Correspondence to P Pantazis

published procedures.[14,15] 9NC suspensions were prepared in polyethylene glycol (PEG 400; Aldrich, Milwaukee, WI).[1-3, 16-18] Other anticancer drugs used in this study include amsacrine (*m*-AMSA; Division of Cancer Treatment, NCI, NIH, Bethesda, MD), etoposide (VePesid; Bristol-Myers, Evansville, IN), daunorubicin (cerubidine; Wyeth Laboratories, Philadelphia, PA), cytarabine (1-β-D-arabinofuranosylcytosine; Cetus, Emerville, CA), methotrexate (methotrexate sodium; Lederle Laboratories, Pearl River, NY), carmustine (BCNU; Bristol-Myers), and vincristine (vincristine sulfate; David Bull Laboratories, Mulgrave, Victoria, Australia). Mechanisms of action, pharmacology, molecular biology, cancer biology and clinical oncology of these drugs are reviewed elsewhere.[19,20]

Cells

Wild-type human leukemia U-937 (U-937/wt) cells were originally obtained from Dr K Nilsson[21] and used to develop 9NC-resistant cells as described.[13] The resulting sublines exhibit various levels of resistance to 9NC and have been designated U-937/CR1, U-937/CR2, etc. The designation CR (for camptothecin-resistant) is followed by a numerical indicating the highest concentration of 9NC, in μM, in the media used to propagate the cells. For example, the designations U-937/CR1 and U-937/CR10 indicate that 1 and 10 μM were the respective highest 9NC concentrations to which the U-937/CR cells were exposed and propagated for 6 months at least prior to use in experimentation. Briefly, studies on partial characterization of various U-937/CR cell sublines have shown that progressive increase in 9NC resistance is associated with appearance of several features in these cells including a decrease in proliferation rates *in vitro*, an increased number of cells with morphological features of granulocytes, an increase in the number of cells that produce superoxide, a decrease in the number of cells that adhere to plastic following treatment with phorbol ester, a decrease in tumorigenic ability when xenografted in nude mice, a decrease in synthesis of topo I and an increase in synthesis of topo II.[13]

Microscopy and flow cytometry

Untreated and drug-treated U-937/wt and U-937/CR cells, maintained as suspension cultures, were pelleted by gentle centrifugation on glass slides, fixed, and stained with Wright-Giemsa (Accustain; Sigma, St Louis, MO). Subsequently, dry preparations of stained cells on slides were covered with immersion oil (type A; Cargille, Cedar Grove, NJ), examined under a Zeiss microscope equipped with a camera and photomicrographed on Kodak 200 film. DNA content of cells was determined by flow cytometry using the EPICS-ELITE Laser Flow Cytometer (Coulter, Hialeah, FL) and analyzed with the aid of the MULTICYCLE program (Phoenix Flow Systems, San Diego, CA). Cell cycle fitting and computation of percentage of cell fractions at specific stages were based upon the polynomial S phase algorithm developed by Dean and Jett,[22] with an interactive, non-linear least squares fit performed by the method of Marquardt.[23] This method of mathematical analysis is the most accurate of the existing methods for evaluation of S phase of an asynchronous cell population (reviewed elsewhere[24]). The methodologies for microscopy and flow cytometry used in the present study have been used in several recent reports.[1,2,16,25,26]

Results

Proliferation of cells treated with various anticancer drugs

In this section, we investigated the effects of various anticancer drugs on the proliferation rates of U-937/wt and U-937/CR cells. We initially tested topo II-directed agents including amsacrine, etoposide and daunorubicin. The cell sensitivity to these drugs was assessed by comparing the proliferation rates of untreated and drug-treated cells. Figure 1 shows results using two different concentrations for each drug. Although all U-937/CR cell sublines were tested, Figure 1 shows the results of U-937/CR1 and U-937/CR10 cells that differ dramatically in 9NC resistance. A 20 nM concentration of amsacrine had a little inhibitory effect on the proliferation rate of U-937/wt cells (Figure 1A), but the effect increased as the 9NC resistance of cells increased (Figure 1B and C). Like amsacrine, etoposide and daunorubicin inhibited cell proliferation with the effect becoming more effective as 9NC resistance of the cells increased. The inhibitory effectiveness of etoposide and daunorubicin was similar on the 9NC-resistant cells (Figure 1B and C), but on the basis of equimolar concentrations, etoposide and daunorubicin were more effective than amsacrine. Further, a higher drug concentration of 60 nM inhibited cell proliferation more than the 20 nM concentration as expected. This can be

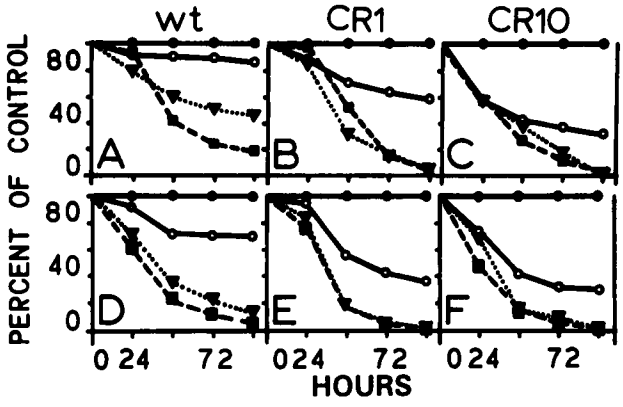

Figure 1. Proliferation of cells treated with topo II-directed drugs. Exponentially proliferating cells were transferred to fresh culture media without (for U-937/wt cells) or with the appropriate 9NC concentration (for U-937/CR1 and U-937/CR10 cells) and adjusted to equal cell density. Cells were counted every 24 h and the cell number was estimated as percent of control, i.e. cells that were not treated with topo II-directed drugs. U-937/wt (A and D), U-937/CR1 (B and E), and U-937/CR10 (C and F) cells were treated with 20 nM (A–C) or 60 nM (D–F) of amsacrine (○), etoposide (▼) or daunorubicin (■).

observed by the slopes of the graphs in Figure 1(D–F). In general, the pattern of cell sensitivity to 60 nM drugs was similar to the sensitivity pattern exhibited in the presence of 20 nM drugs, i.e. the sensitivity increased as the 9NC resistance increased.

In addition to topo II-directed drugs, the cells were treated with anticancer drugs that do not interfere with topoisomerase activities including cytarabine, methotrexate, carmustine and vincristine. At a concentration as high as 200 nM, cytarabine and carmustine did not alter the proliferation rates of the U-937/wt and U-937/CR cells, whereas both cell lines were highly sensitive to methotrexate or vincristine concentrations as low as 5 or 2 nM, respectively, i.e. the minimal drug concentrations required to completely inhibit cell proliferation (graphs of proliferation rates not shown). In general, drugs that do not interfere with topoisomerase activities elicited similar responses from the cells regardless of the extent of 9NC resistance. In other words, both U-937/wt and U-937/CR cells were either insensitive or similarly sensitive to the drug.

Microscopy of drug-treated cells

Studies of proliferation rates were accompanied by microscopy observations of the cells treated with the various drugs. Microphotographs representing random slide areas with cells subjected to treatments are shown in Figure 2. U-937/wt cells appear unaffected after a 48 h period of treatment with daunorubicin, with less than 2% of apoptotic cells present (Figure 2A). In contrast, U-937/CR10 cells are more sensitive to the same daunorubicin concentration with 15–20% of the daunorubicin-treated U-937/CR10 cells exhibiting typical apoptotic features, i.e. masses of fragmented, highly heterochromatic chromatin (Figure 2B). Also, treatment of U-937/CR10 cells with another topo II-directed drug, etoposide, results in appearance of apoptotic cells (Figure 2C) at a frequency similar to the daunorubicin-treated cells. Further, vincristine and methotrexate induce different morphological changes in the U-937/wt and 9NC-resistant cells. The effects of low concentration of vincristine on the cells are shown in Figure 2(D–F). The vincristine-induced changes vary in U-937/wt cells, i.e. some cells are unaffected, other cells exhibit highly aggregated but not segmented chromatin, whereas other cells exhibit typical apoptotic appearance with several individual, well-fragmented chromatin masses (Figure 2D). This variability in morphological features exhibited by the cells may correlate with the heterogeneity of cell subpopulations comprising the U-937/wt cell line. However, there is much less variability in the morphological features of vincristine-treated U-937/CR1 and U-937/CR10 cells,

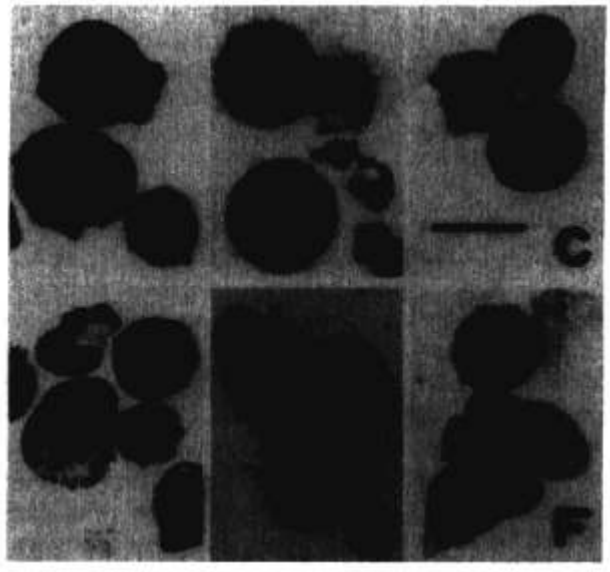

Figure 2. Microphotography of cells treated with various anticancer drugs. U-937/wt (A and D), U-937/CR1 (E) and U-937/CR10 (B, C and F) cells were exposed for 48 h to 10 nM daunorubicin (A and B), 10 nM etoposide (C) and 5 nM vincristine (DF), then cytospin pelleted on slides and stained. Bar=20 nm (C).

which exhibit highly aggregated chromatin (Figure 2E and F), with the chromatin aggregate in U-937/CR10 cells showing a smoother perichromatin formation (Figure 2E).

Perturbations in the cell cycle of cells treated with topo II-directed drugs

Flow cytometry was used to monitor perturbations in the cell cycle of drug-treated cells as a result of changes in the relative DNA content of the cells. The extent of perturbations induced by the topo II-directed drugs amsacrine, daunorubicin and etoposide was investigated at low and high drug concentrations. Previous *in vitro* studies with normal and malignant cells derived from solid tissues have indicated that the pattern and extent of cell cycle perturbation depend on the drug concentration in the cell culture.[16] Figure 3 shows histograms of DNA content in U-937/wt, U-937/CR1 and U-937/CR10 cells exposed to a low drug concentration of 20 nM for 96 h. There is a marked difference in the sensitivity of these cells to the anticancer drugs tested at equimolar concentrations. Amsacrine has no apparent effect on the cell cycle of the cells, since the histogram profiles of the untreated (Figure 3A, E and I) and drug-treated (Figure 3B, F and J)

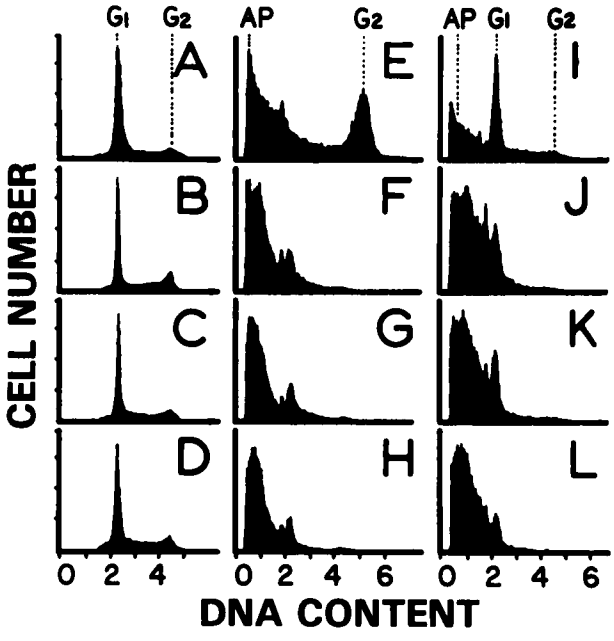

Figure 4. DNA content analysis of cells treated with high drug concentration. U-937/wt (A, E and I), U-937/CR1 (B, F and J), U-937/CR4 (C, G and K) and U-937/CR10 (D, H and L) cells were treated for 96 h with 200 nM etoposide (E–H) or daunorubicin (I–L), whereas controls received no treatment (A–D). Relative DNA content was determined by flow cytometry. G_1, $G_0 + G_1$ cells; G_2, $G_2 + M$ cells; and AP, apoptotic cells.

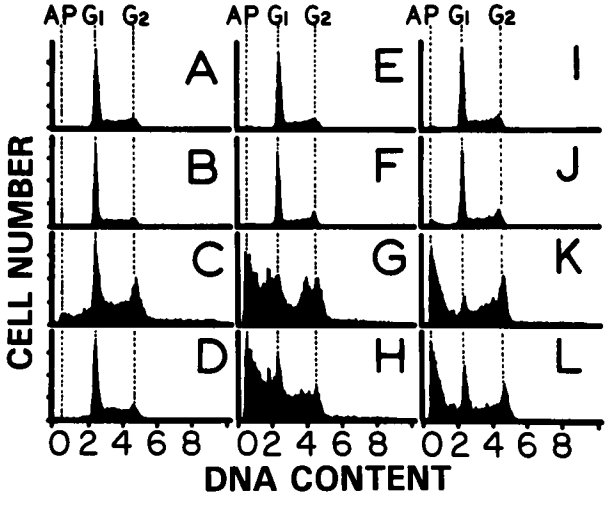

Figure 3. DNA content analysis of cells treated with low drug concentration. U-937/wt (A–D), U-937/CR1 (E–H), and U-937/CR10 (I–L) cells were exposed for 96 h to amsacrine (B, F and J), daunorubicin (C, G and K), etoposide (D, H and L) or received no treatment (controls; A, E and I). Relative DNA content was determined by flow cytometry. G_1, $G_1 + G_0$ cells; G_2, $G_2 + M$ cells; and AP, apoptotic cells.

cells are similar. In contrast, both daunorubicin and etoposide introduce perturbations in the cell cycle of the cells. Specifically, the histograms indicate that U-937/CR1 (Figure 3G and K) and U-937/CR10 (Figure 3H and L) cells are more sensitive than U-937/wt cells (Figure 3C and D) to these drugs. Further, U-937/CR10 cells are more sensitive than U-937/CR1 cells to the drugs as indicated by the lower number of cells in the G_1 and G_2 phases.

Another measure of cell sensitivity to anticancer drugs is the extent of cell death induced upon treatment with high equimolar drug concentrations. Figure 4 shows histograms of DNA content in cells exposed to 200 nM of etoposide and daunorubicin for 96 h. These histograms were utilized to estimate the percentage of fractions corresponding to apoptotic cells (Table 1). The drugs induce apoptosis (AP peak) in 60–70% of the U-937/wt cells and in more than 90% of U-937/CR cells. In general, the histograms indicate that the cell sensitivity to etoposide and daunorubicin increases concomitantly with the increase of resistance to 9NC (Figure 4).

Table 1. Percentage of apoptotic cells in cultures

Cells	Treatment		
	untreated (control)	+ eptoposide for 96 h	+ daunorubicin for 96 h
U-937/wt	3.5 (A)	61 (E)	71 (I)
U-937/CR1	4.0 (B)	92 (F)	90 (J)
U-937/CR4	2.5 (C)	92 (G)	93 (K)
U-937/CR10	2.0 (D)	90 (H)	97 (L)

Percentage of apoptotic fraction was estimated in each cell culture by flow cytometric analysis of DNA content as described in Materials and methods. Letters in parentheses indicate the histogram in Figure 4 used to estimate the percentage. Small variations in percentages were estimated in three repeated experiments, but the patterns of histograms and, therefore apoptotic fractions, were similar to the results shown in Figure 4 and Table 1.

Perturbations in the cell cycle of cells treated with various anticancer drugs

In addition to topo II-directed drugs, we examined changes in fractions of U-937/wt and U-937/CR cells treated with anticancer drugs that their mechanism of action does not interfere with topoisomerase activities. These drugs include the antimetabolites cytarabine and methotrexate, the nitrosourea carmustine, and the mitotic inhibitor vincristine. The cells were exposed to 200 nM of each drug for 96 h and then subjected to flow cytometry analysis of DNA content (Figure 5). Cytarabine had no detectable effect on the cell cycle of the U-937/wt (Figure 5A2) and U-937/CR (Figure 5B2 and C2) cells, as judged by the similarity to the histograms of the untreated cells (Figure 5A1–C1). Similarly, carmustine (BCNC) did not introduce detectable permutations in the cell cycle of these cells (Figure 5A–C4). In contrast, methotrexate and vincristine dramatically affected the cell cycle of the cells (Figure 5A3–C3, A5–C5). Unlike methotrexate, however, vincristine resulted in the appearance of U-937/wt cells with DNA content higher (i.e. hyperdiploid cells) than cells in G_2M as indicated by the arrow in Figure 5(A5). No hyperdiploid cells were detected by flow cytometry in vincristine-treated U-937/CR1 (Figure 5B5) and U-937/CR10 (Figure 5C5) cells. Moreover, vincristine-treated cultures of U-937/CR10 cells contained virtually only apoptotic cells (Figure 5C5), whereas vincristine-treated cultures of U-937/CR1 cells contained small but detectable fractions of cells at G_1 and G_2 (Figure 5B5).

Further, by applying decreased concentrations of etoposide and daunorubicin, it was determined that a concentration of 10 nM was efficient for both drugs to induce perturbations in the cell cycle of U-937/CR cells (results not shown), but not in the cell cycle of U-937/wt cells (Figure 6B and C). Finally, it is of interest that low vincristine concentrations, 20 nM or lower, consistently resulted in appearance of a larger hyperdiploid fraction in U-937/wt than in U-937/CR cells.

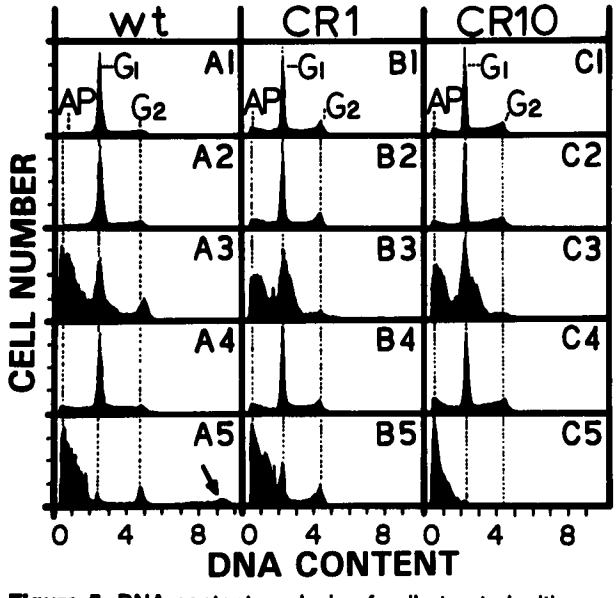

Figure 5. DNA content analysis of cells treated with various anticancer drugs. U-937/wt (A1–A5), U-937/CR1 (B1–B5), and U-937/CR10 (C1–C5) cells were treated for 96 h with 200 nM cytarabine (A2, B2, C2), 200 nM vincristine (A3, B3 and C3), 200 nM BCNU (A4, B4 and C4), and 200 nM methotrexate (A5, B5 and C5) or received no drug treatment (A1, B1 and C1). Arrow indicates the peak corresponding to hyperdiploid cells.

Discussion

In view of the use of the water-insoluble CPT and some CPT-derivatives in ongoing clinical trials, it is likely that some cancer patients will become unresponsive to these drugs because of development of resistance. Within this context, we have presented evidence that development of cell sublines with increased 9NC resistance is associated with appearance of several features in these cells.[12,13,27] Like the parental cells, U-937 cells resistant to 9NC concentrations of 0.5 μM or less are tumorigenic in nude mice,[27] whereas U-937 cells resistant to 9NC concentrations of 1 μM or higher have lost the ability to induce tumors in nude mice.[13] Other studies of parental U-937/wt cells and low 9NC resistane U-937/CR0.15 cells have shown that they (i) have similar proliferation rates *in vitro*; (ii) express

similar levels of topo I mRNA; (iii) synthesize proteins of 100, 75, and 67 kDa that react with anti-topo I serum; (iv) demonstrate similar levels of topo I catalytic activity as determined by relaxation assays of supercoiled plasmid DNA in absence of 9NC, whereas in presence of 9NC topo I activity from U-937/CR0.15 cells is approximately 10-fold more resistant that that from U-937/wt cells; (v) differ in extent of topo I gene methylation, with the U-937/CR0.15 topo I being hypermethylated; (vi) differ in the nucleotide sequence of topo I cDNAs at position 361, resulting in presence of serine or phenylalanine in topo I of U-937/wt or U-937/CR0.15 cells, respectively; and (vii) differ in their sensitivity to topo II-directed drugs, etoposide and daunorubicin, with the U-937/CR0.15 cells exhibiting higher sensitivity.[12,13,27] Further, U-937 cells resistant to 9NC concentrations of 1 μM or higher exhibit (i) proliferation rates slower than those of U-937/wt and U-937/CR0.15 cells; (ii) morphological and functional features of granulocytic maturation; (iii) altered sensitivities to physiological and non-physiological agents that induce leukemia cell maturation; (iv) decreased or virtually no synthesis of topo I; and (v) increased synthesis of topo II.

The exact events and/or mechanisms that have resulted in cells resistant to 9NC are not completely understood, but it appears that development of resistance to 9NC does not correlate with synthesis of P-glycoprotein that confers multidrug resistance to cells.[12] Taken together, the findings described previously[12,13,27] and in this report clearly indicate that development of low and high resistance to 9NC correlates with alteration and decreased expression of the topo I gene, respectively, in malignant cells. Ultimately, complete insensitivity to 9NC may correlate with absolute dependence of the cell growth on topo II synthesis. In this context, it has been hypothesized that there exists a compensatory interaction between topo I and topo II enzymes in the mammalian cells, i.e. a decrease in activity or synthesis of one topo enzyme is accompanied by increase in activity or synthesis of the other topo enzyme.[8,28,29] The clinical importance of the findings presented in this report is that, in addition to its direct action of 9NC in cell killing, development of 9NC resistant cells and tumors may be accompanied by a concomitant hypersensitivity to topo II-directed chemotherapeutic agents.

This hypersensitivity of 9NC resistant U-937 cells to topo II-directed agents prompted us to investigate sensitivity to other agents currently used alone or in combination to treat cancer patients. The nitrosourea carmustine had no detectable effect on both parental and 9NC-resistant cells perhaps because the drug degrades rapidly[19] in the media before exerting any effect. The vinca alkaloid vincristine was more effective on the 9NC resistant than on parental cells in inducing cell cycle perturbations and apoptosis at low and high drug concentrations, respectively. However, vincristine results in the appearance of a hyperdiploid cell fraction in cultures of U-937/wt, but not U-937/CR cells. Presumably, the hyperdiploid cells die afterwards. It is possible that appearance of the hyperdiploid fraction correlates with topo I synthesis or activity, but we have no experimental support for this hypothesis.

Finally, the high 9NC-resistant cells were tested for sensitivity to the anticancer drugs cytarabine and methotrexate that inhibit key enzymes in the purine or pyrimidine synthetic pathways and thus interfering with DNA synthesis or the S phase of the cell cycle.[19,20] The responses of the cells were different to these two antimetabolites used at an equimolar concentration of 200 nM. Cytarabine had no detectable effect on both the parental and 9NC-resistant cells as judged by absence of perturbations in the cell cycle of the cells, in agreement with a previous report that this concentration, unlike higher concentrations, does not induce internucleosomal DNA fragmentation in U-937 cells.[30] However, an equimolar methotrexate concentration of 200 nM was more effective on U-937/CR10 than U-937/wt cells in induction of apoptosis. Therefore, it appears that acquisition of high 9NC resistance by the cells renders them more sensitive to methotrexate.

In conclusion, development of 9NC resistance by U-937 cells is accompanied by increased sensitivity to drugs already used in cancer chemotherapy including drugs of diverse origin such as the topo II-directed drugs amsacrine, etoposide and daunorubicin, the vinca alkaloid vincristine, and the antimetabolite methotrexate. Increased sensitivity of the 9NC-resistant U-937 cells to topo II-directed drugs correlates well with increased synthesis of topo II, but increased sensitivity to vincristine and methotrexate has not been correlated yet with a change in a specific event or mechanism. In general, this increased cell sensitivity to some known anticancer drugs may have clinical implications for the treatment of cancer patients who become resistant to 9NC.

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