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Cell cycle phase specificity in the potentiation of etoposide-induced DNA damage and apoptosis by KN-62, an inhibitor of calcium-calmodulin-dependent enzymes

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Abstract

The cell cycle phase-dependent induction of DNA damage and apoptosis by etoposide (VP-16) and its modulation by 1-[*N*,*O*-bis(1,5-isoquinolinesulfonyl)-*N*-methyl-l-tyrosyl]-4-piperazine (KN-62), an inhibitor of calcium-calmodulin-dependent enzymes, were examined in sensitive (HL-60/S) and VP-16-resistant (HL-60/DOX0.05) HL-60 cells. Cells from exponential-phase cultures were enriched by centrifugal elutriation into G_1, S, and G_2+M fractions. Modulation of VP-16-induced apoptosis by KN-62 in HL-60/S cells was apparent only in the S phase at the IC_{50} concentration. However, in the HL-60/DOX0.05 cells, significant ($P < 0.001$) potentiation of VP-16-induced apoptosis by a non-cytotoxic concentration of 2 μM KN-62 was apparent in cells in the G_1, S, and G_2+M phases, as well as over the entire concentration range tested. VP-16-induced apoptosis and its potentiation by a non-cytotoxic concentration of 2 μM KN-62 were correlative with drug-stabilized DNA cleavable complex formation based on a band depletion assay. In agreement with the results on apoptosis in the resistant HL-60/DOX0.05 cells, the enhanced depletion of the α and β isoforms of topoisomerase II by VP-16 + KN-62 was observed in G_1, S, and G_2+M cells. Results suggest that the effects of KN-62 in reversing resistance are based on its role as a potent sensitizer of VP-16-induced DNA damage and apoptosis in a cell cycle phase-independent manner.

Keywords: Apoptosis; Etoposide; Drug resistance; Topoisomerase II; Calcium-calmodulin

1. Introduction

The topoisomerases, which alter DNA topology for the processing of genetic material, are also targets for clinically important anti-neoplastic agents [1–4]. Topoisomerase I (topo I), which is a 97 kDa protein, is the target for camptothecin and its analogs [3,4]. In mammalian cells, topoisomerase II (topo II), which exists as the α (170 kDa) and β (180 kDa) isoforms [1,2,5], is the target for clinically active anthracyclines and epipodophyllotoxins. Although substantial literature exists on the regulation, function, and role in drug resistance of the α isoform, mechanisms governing sensitization of VP-16-resistant cells and the potential involvement of the β isoform have yet to be elucidated [1–4].

Topo IIβ appears to have an essential role in cell differentiation [6,7]. However, the recent data of Herzog *et al.* [8] suggest that the absence of topo IIβ does not interfere with cell proliferation. This and other studies provide evidence that tumor cell resistance to *m*-AMSA, the DNA intercalating topo II poison, may functionally involve topo IIβ [9,10]. Also, the differential interaction of *m*-AMSA and VP-16 with the topo II isoforms may contribute to altered drug resistance profiles [11].

Tumor cells selected for resistance to DOX exhibit overexpression of P-glycoprotein and alterations in drug-stabilized DNA cleavable complex formation but not protein levels of topo II. While the site-specific hypophosphorylation of topo IIα is correlative with the reduced DNA damage, we also have found that, unlike topo IIα, the overall and site-specific hyperphosphorylation of topo IIβ is markedly higher in the HL-60/DOX0.05 compared with the HL-

Abbreviations: VP-16, etoposide; DOX, doxorubicin; *m*-AMSA, amsacrine

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60/S cells [11,12]. It has been demonstrated that while the protein levels and phosphorylation of topo IIα are cell cycle phase regulated, protein levels of topo IIβ do not exhibit similar alterations [1,2,6,7]. Also in the resistant cells, inhibitors of calcium-calmodulin-dependent enzymes, e.g. 1-[*N,O*-bis(1,5-isoquinolinesulfonyl)-*N*-methyl-l-tyrosyl]-4-piperazine (KN-62) [13], are markedly effective in potentiating VP-16-induced apoptosis. We thus investigated in sensitive (HL-60/S) and resistant (HL-60/DOX0.05) HL-60 cells the cell cycle phase-dependent sensitization of VP-16-induced drug-stabilized DNA cleavable complex formation and apoptosis by KN-62. The data suggest that the sensitization of VP-16-induced DNA damage and apoptosis in resistant cells by KN-62 occurs in the G_1, S, and G_2+M phases of the cell cycle. Also in the presence of KN-62, there is a marked increase of VP-16-stabilized topo II DNA cleavable complex formation with the α and β isoforms of topo II.

2. Materials and methods

The wild-type HL-60 (HL-60/S) cells were obtained from Dr. Andrew Yen, College of Veterinary Medicine. Cultures of HL-60/S cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 2 mM L-glutamine (BioWhittaker) at 37° in a humidified 5% CO_2 plus 95% air atmosphere. The resistant subline of HL-60 developed by culturing the wild-type cells in increasing concentrations of 0.025 to 0.05 μg/mL of DOX has been described previously [12]. Following *in vitro* selection, the DOX-resistant subline (HL-60/DOX0.05) was routinely cultured in the absence of DOX. Doubling time *in vitro* of the HL-60 and HL-60/DOX0.05 cells was 18–20 hr. Based on drug exposure for 1–3 hr and cell kill assessed by a soft-agar colony assay, the HL-60/DOX0.05 cells are 40-fold resistant to the cytotoxic effects of VP-16 compared with the HL-60/S cells [12].

The enrichment of cells in the G_1, S, and G_2+M phases of the cell cycle was carried out by centrifugal elutriation [14] in a J2–21 centrifuge equipped with a JE-6 rotor (Beckman-Coulter). Briefly, cells (2×10^8) were loaded at a rotor speed of 2000 and 1875 rpm for the HL-60/S and HL-60/DOX0.05 cells, respectively. The flow rate of the medium (RPMI 1640 supplemented with 2 mM L-glutamine and 10% newborn calf serum) was maintained at 10 mL/min during loading of the cells. Fractions of cells (50 mL) were collected following changes in the flow rate in increments of 1–2 mL. In a typical run, 16–18 fractions were collected. The cells in each fraction were collected by centrifugation at 150 *g* and analyzed for cell cycle phase distribution by flow cytometry [13]. Fractions in the cell cycle phase of interest were pooled and used for experiments analyzing the effects of KN-62 on VP-16-induced apoptosis.

Cells were treated with 0.5 to 40 μM VP-16 in the absence or presence of 2 μM KN-62 for 1 hr at 37°, washed, and resuspended in drug-free medium. Samples retrieved at 4 hr were processed for apoptosis staining in cytospin preparations with terminal transferase and fluorescein-12-dUTP (Boehringer Mannheim) as described earlier [13,15]. A minimum of 200 cells in at least three separate fields were counted using a fluorescence microscope to determine the percentage of apoptotic cells [13,15].

The topo II isoform specific enhancement by VP-16 of topo II–DNA cleavable complex formation was determined by the band depletion technique [11,13]. The HL-60/S or HL-60/DOX0.05 cells were enriched in the G_1, S, and G_2+M phases of the cell cycle by elutriation and treated with VP-16 ± KN-62 for 1 hr at 37°. Control and treated cells (2×10^6) were lysed in 2x Laemmli buffer [13]. The samples were processed by SDS–PAGE [12,13] and electroblotted onto nitrocellulose [13], and topo II was detected with an antibody that specifically recognizes the 170 or 180 kDa isoform [11,13,15]. Topoisomerase II protein levels in control and treated cells were detected by chemiluminescence (Pierce) or by using ^{125}I-goat anti-rabbit IgG. Depletion of topo II due to enhanced cleavable complex formation in treated versus control cells was quantified by a PhosphorImager.

3. Results and discussion

The enrichment of HL-60/S or HL-60/DOX0.05 cells in the G_1, S, and G_2+M phases of the cell cycle by centrifugal elutriation is shown in Fig. 1. In a typical experiment, the distribution of cells in the G_1, S, and G_2+M phase was 43, 44, and 13%, respectively, in asynchronous exponential cultures of HL-60/S or HL-60/DOX0.05 cells. Following elutriation, the enrichment of cells in the G_1, S, and G_2+M phases was ~95, ~82, and ~68%, respectively.

KN-62, an inhibitor of calcium-calmodulin-dependent enzymes, sensitizes HL-60/DOX0.05 cells to the cytotoxic effects of VP-16 by enhancing DNA damage and inducing apoptosis [13]. Analysis of these treated cells by flow cytometry has revealed that cell depletion due to apoptosis is maximal in the S phase [13]. To understand the mechanistic basis for this selectivity, the cell cycle phase-dependent effects of VP-16 in the absence or presence of KN-62 were determined in HL-60/S and HL-60/DOX0.05 cells (Fig. 2, Tables 1 and 2). As shown in Fig. 2, in HL-60/S and HL-60/DOX0.05 cells treated with the IC_{80} and IC_{50} concentrations of VP-16, respectively, the apoptotic response was enhanced in the presence of 2 μM KN-62. As outlined in Table 1, maximal VP-16-induced apoptosis was apparent in the S phase of HL-60/S cells. Further, a non-cytotoxic concentration of 2 μM KN-62 had a significant effect (P = 0.005) in enhancing the apoptotic effects in S-phase cells treated with 1 or 2.5 μM VP-16 and in G_2+M cells treated with 2.5 μM VP-16. In contrast to these results with HL-60/S cells (Table 1), the non-cytotoxic concentration of 2 μM KN-62 significantly enhanced (P = 0.001) VP-16-induced apoptosis in the G_1, S, or G_2+M phases of HL-60/

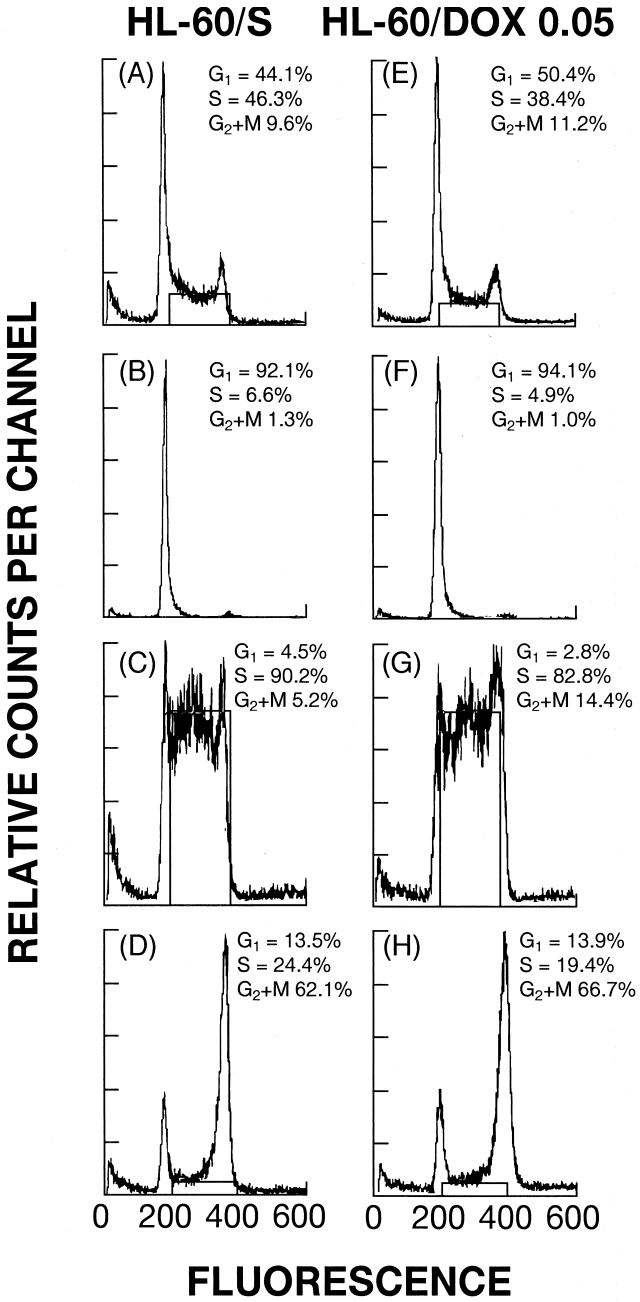

Fig. 1. Analysis of cell cycle phase distribution in HL60/S (A–D) and HL-60/DOX0.05 (E–H) separated by centrifugal elutriation. The cell cycle phase distribution in asynchronous exponential cultures of HL-60/S and HL-60/DOX0.05 cells are shown in panels A and E, respectively. Cells enriched in the G_1, S, and G_2+M phases from HL-60/S cells are in panels B, C, and D, respectively. Cells enriched in the G_1, S, or G_2+M phases from HL-60/DOX0.05 cells are in panels F, G, and H, respectively. The elutriation procedure has been repeated on at least 6–8 different occasions with similar enrichment of cells, and the data presented are from a representative experiment.

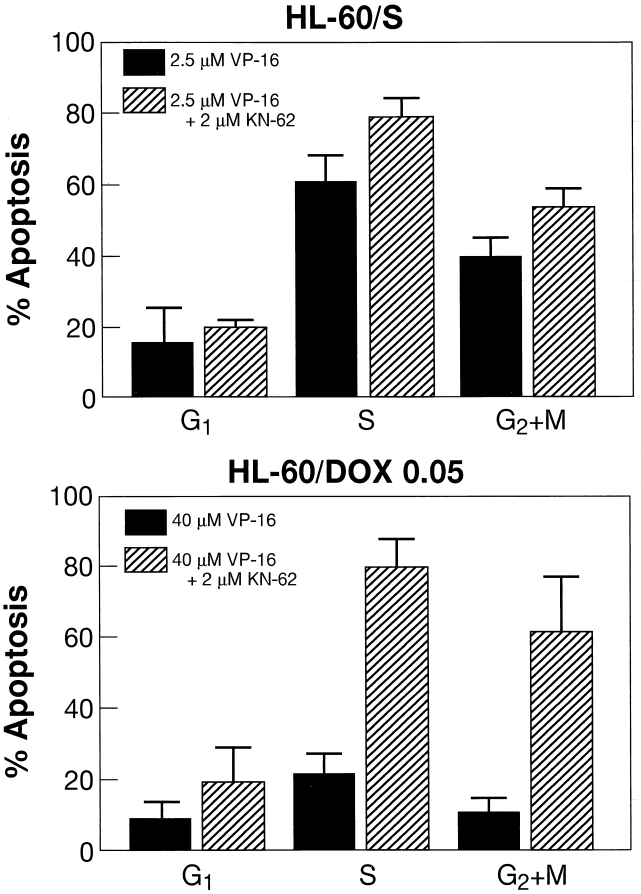

Fig. 2. Apoptotic response following treatment with the IC_{50} concentration of VP-16 in the absence or presence of 2 μM KN-62 in G_1, S, and G_2+M enriched populations obtained by centrifugal elutriation of HL-60/S and HL-60/DOX0.05 cells. Cytospin preparations from samples of control or treated cells retrieved at 4 hr were processed for apoptosis staining with terminal transferase and fluorescein-12-dUTP. Data are means ± SD from triplicate experiments.

DOX0.05 cells (Table 2). Also, the potentiating effects of KN-62 on apoptosis were apparent over the entire range of VP-16 concentrations tested.

Since KN-62 significantly affected VP-16-induced apoptosis in HL-60/DOX0.05 cells, we tested the effects on VP-16-stabilized topo II–DNA cleavable complex formation as a measure of induced DNA damage. The results from the band depletion assay shown in Fig. 3 demonstrate that depletion of the α and β isoforms of topo II by VP-16 alone was maximal in the S phase for HL-60/S cells. A marked increase in the depletion of the α and β isoforms of topo II in HL-60/S cells was also apparent in G_2+M cells treated with VP-16 + KN-62. In contrast, cell cycle phase specificity was apparent in the detectable depletion of the α or β isoform of topo II by VP-16 alone in the HL-60/DOX0.05 cells enriched in the S and G_2+M phases. However, in the presence of a non-cytotoxic concentration of 2 μM KN-62, a marked depletion of the α and β isoforms of topo II was apparent in cells enriched in the G_1, S, and G_2+M phases of the cell cycle.

We have reported previously the effects of KN-62 on hyperphosphorylation and the enhanced depletion of topo IIα by VP-16 [13]. Since the present data suggest the potent sensitizing effects of KN-62 on VP-16-induced apoptosis, the effect of such treatment on drug-stabilized DNA cleav-

Table 1
Cell cycle phase-dependent effects of KN-62 on VP-16-induced, apoptosis in HL-60/S

Treatment[a]	cells		
	% Apoptosis[b]		
	G_1	S	G_2+M
Control or 2 μM KN-62	4.3 ± 2.6[c]	6.4 ± 3.0	7.4 ± 0.5
0.5 μM VP-16	4.2 ± 1.5	9.2 ± 2.4	10.0 ± 3.0
0.5 μM VP-16 + 2 μM KN-62	6.4 ± 1.2	9.7 ± 2.0	14.3 ± 3.4
1.0 μM VP-16	7.1 ± 3.6	14.0 ± 5.6	11.4 ± 3.1
1.0 μM VP-16 + 2 μM KN-62	10.0 ± 4.7	28.6 ± 4.6*	17.5 ± 8.8
2.5 μM VP-16	15.2 ± 9.6	60.6 ± 7.0	39.1 ± 5.4
2.5 μM VP-16 + 2 μM KN-62	19.0 ± 2.4	78.8 ± 4.8*	52.0 ± 5.4*

[a] Cells enriched in the different cell cycle phases were treated with the indicated concentrations of VP-16 ± 2 μM KN-62 for 1 hr, washed, and resuspended in drug-free medium.
[b] Apoptosis was determined at 4 hr following staining with fluorescein-12-dUTP [13,15].
[c] Values are means ± SD from at least triplicate experiments.
* Significantly different ($P = 0.005$) from the control or treatment with a similar concentration of VP-16 in the absence of 2 μM KN-62.

able complex formation with phosphorylated topo IIβ was determined. Compared with cells in the G_1 or G_2+M fraction, in the elutriated S-phase fraction from HL-60/DOX0.05 cells treated with 40 μM VP-16 the depletion of topo IIβ protein was 1.7-fold higher in the presence of 2 μM KN-62 (data not shown).

Tumor cell resistance to topo II poisons generally is attributed to reduced drug accumulation mediated by P-glycoprotein or a decrease in the protein level of topo IIα [3]. In general, compared with the G_1 and G_2+M phases, cells in the S phase are more sensitive to the DNA damaging and cytotoxic effects of VP-16 and DOX. Our previous studies with the HL-60/DOX0.05 cells have demonstrated that resistance to VP-16 cannot be explained by alterations in the steady-state level of topo II protein or decreased drug accumulation [12,13]. Further, the reversal of VP-16 resistance by KN-62 was due to a specific enhancement of topo II–DNA cleavable complex formation and apoptosis [13]. Since topo II has two distinct isoforms, a role for the β isoform revealed hyperphosphorylation of the protein in the HL-60/DOX0.05 compared with the HL-60/S cells [11]. These observations prompted us to systematically evaluate the cell cycle phase specificity for VP-16 effects on apoptosis and the interaction with topo IIβ in the absence or presence of KN-62.

Inhibitors of calcium-calmodulin-dependent enzymes are potent sensitizers of DOX and VP-16 cytotoxicity in resistant cells [13]. The efficacy of these sensitizers is dependent upon intracellular free calcium transients [13,16,17], and their ability to reverse resistance is not due to restoration of defects in drug accumulation [13,17]. We had suggested previously that the potentiation of VP-16-induced apoptosis by KN-62 is targeted to cells in the S phase [13]. The present data further support this finding in both the HL-60/S

Table 2
Cell cycle phase-dependent effects of KN-62 on VP-16-induced, apoptosis in HL-60/DOXO.05 cells

Treatment[a]	cells		
	% Apoptosis[b]		
	G_1	S	G_2+M
Control or 2 μM KN-62	2.7 ± 0.3[c]	5.6 ± 1.7	7.5 ± 1.8
5.0 μM VP-16	4.3 ± 1.6	5.3 ± 1.3	6.1 ± 2.4
5.0 μM VP-16 + 2 μM KN-62	9.4 ± 6.6	33.7 ± 8.6*	10.9 ± 4.2
10.0 μM VP-16	4.1 ± 1.7	5.2 ± 0.7	5.6 ± 2.7
10.0 μM VP-16 + 2 μM KN-62	13.0 ± 7.1*	60.1 ± 10.9*	21.7 ± 6.6**
40.0 μM VP-16	8.6 ± 4.4	22.0 ± 6.1	11.3 ± 3.5
40.0 μM VP-16 + 2 μM KN-62	19.5 ± 9.3*	79.4 ± 7.6*	61.9 ± 15.8*

[a] Cells enriched in the different cell cycle phases were treated with the indicated concentrations of VP-16 ± 2 μM KN-62 for 1 hr, washed, and resuspended in drug-free medium.
[b] Apoptosis was determined at 4 hr following staining with fluorescein-12-dUTP [13,15].
[c] Values are means ± SD from at least triplicate experiments.
* Significantly different ($P = 0.001$) from the control or treatment with a similar concentration of VP-16 in the absence of 2 μM KN-62.
** Significantly different (P 0.026 and P 0.013) from the control or treatment with a similar concentration of VP-16 in the absence of 2 μM KN-62, respectively.

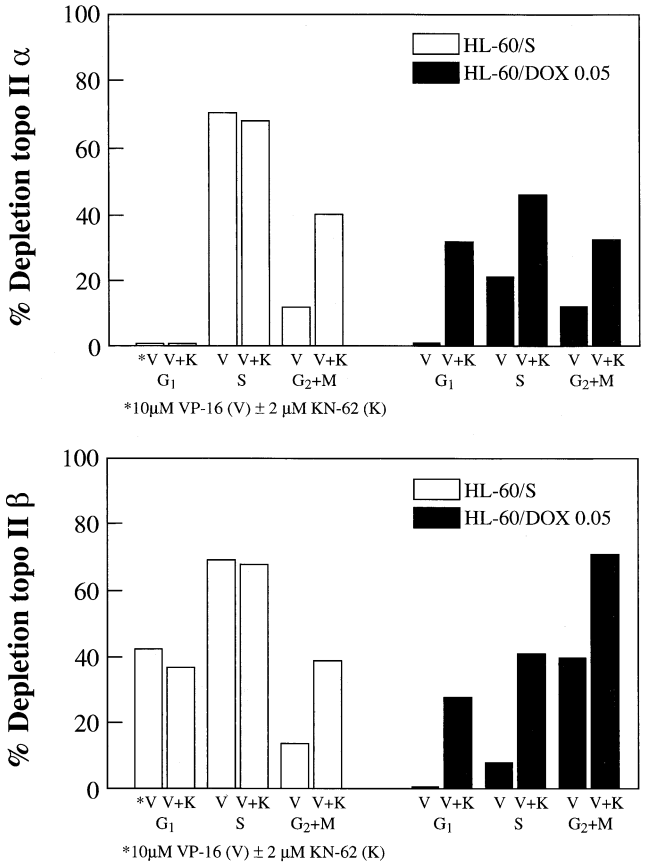

Fig. 3. Band depletion analysis of α (170 kDa) and β (180 kDa) isoforms of topo II in HL-60/S and HL-60/DOX0.05 cells enriched in the G_1, S, or G_2+M phases by centrifugal elutriation and treated with VP-16 ± 2 μM KN-62 for 1 hr. Samples of control and treated cells (2 x 10^6) were lysed in 2x Laemmli buffer, processed by SDS–PAGE, and electroblotted onto nitrocellulose, and topo II was detected with an antisera that specifically recognizes the 170 or 180 kDa isoform. Topoisomerase II protein levels in control and treated cells were detected by chemiluminescence or by using ^{125}I-goat anti-rabbit IgG. Depletion of topo II isoforms was determined by PhosphorImager analysis, and data are mean values from at least duplicate experiments.

and HL-60/DOX0.05 cells. Also of interest with the HL-60/DOX0.05 cells is the enhancement of VP-16-induced apoptosis by KN-62 in the G_1, S, and G_2+M phases. This level of broad activity may be responsible for the ability of KN-62 to reverse resistance to topo II poisons. The effects of KN-62 on the phosphorylation of topo IIα have been reported previously [13], and were suggested to be mechanistically involved in the topo II–DNA cleavable complex formation. The effect of KN-62 on topo II phosphorylation and the potential involvement in DNA cleavable complex formation are also supported by data on the enhanced depletion of the α and β isoforms of topo II and of phosphorylated topo IIβ in HL-60/DOX0.05 cells treated with 40 μM VP-16 + 2 μM KN-62. These results also suggest that the phosphorylated form of topo II, and particularly the β isoform, is mechanistically involved in the drug-stabilized topo II–DNA cleavable complex formation and the subsequent apoptotic response. Overall, the effects of KN-62 on topo II phosphorylation [13], and in stimulating VP-16-stabilized DNA cleavable complex formation with the α and β isoforms, are responsible for the significant level of apoptosis in the treated cells as well as the reversal of resistance.

In summary, results from this study demonstrated that the sensitization to VP-16-induced DNA damage and apoptosis in HL-60/DOX0.05 cells by KN-62, an inhibitor of calcium-calmodulin-dependent enzymes, occurs in the G_1, S, and G_2+M phases of the cell cycle. Ongoing studies are focused on identifying the signaling events in the processing of DNA damage to apoptosis/cell kill. Future studies on tumor cell resistance to topo II poisons should be directed towards an understanding of the drug interaction and regulation of the α and β isoforms of topo II. The identification of signaling cascades involved in apoptosis and cell kill following DNA damage should also contribute to the possible development of novel therapeutic strategies.

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