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Cellular Events Involved in the Sensitization of Etoposide-Resistant Cells by Inhibitors of Calcium-Calmodulin-Dependent Processes

ROLE FOR EFFECTS ON APOPTOSIS, DNA CLEAVABLE COMPLEX, AND PHOSPHORYLATION

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ABSTRACT. Inhibitors of calcium-calmodulin-dependent processes, 1-[*N*,*O*-bis(1,5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-piperazine KN-62 and trifluoperazine (TFP), at non-cytotoxic concentrations (2 and 5 μM, respectively) enhanced etoposide (VP-16) cytotoxicity in Adriamycin®-resistant (HL-60/ADR0.05) cells (3- to >50-fold). In contrast to TFP, the inhibitor KN-62 was able to reverse resistance in HL-60/ADR0.05 cells at VP-16 concentrations that produced equivalent cytotoxicity in sensitive (HL-60/S) cells. Unlike TFP, the cellular accumulation of VP-16 in the presence of KN-62 was enhanced 1.5- to 2-fold in HL-60/S (MDR1 −ve) and HL-60/ADR0.05 (MDR1 +ve) cells. To achieve equivalent cytotoxicity, levels of VP-16 in the resistant cells were >4-fold lower in the presence of KN-62 compared with treatment with VP-16 alone. The sensitizing effects of both KN-62 and TFP were due to enhancement (2- to 4-fold) of VP-16-induced topoisomerase II (TOPO II)-mediated DNA cleavable complex formation, and depletion of the 170 kDa (α) TOPO II isoform. The DNA damage induced by VP-16 in the presence of KN-62 or TFP resulted in the rapid induction of apoptosis and depletion of cells in "S" phase of the cell cycle. Both 5 μM TFP and 2 μM KN-62 enhanced the phosphorylation of 170 kDa TOPO II 1.6-fold and 1.5-fold, respectively. Results suggest that the inhibitory effect of KN-62 or TFP on calcium-calmodulin-dependent processes may be mechanistically involved in sensitizing resistant cells to VP-16 by enhancing TOPO II-mediated DNA damage. *Copyright © 1996 Elsevier Science Inc.* BIOCHEM PHARMACOL 52;12:1903–1909, 1996.

KEY WORDS. topoisomerase II; calcium-calmodulin; etoposide; drug-resistance; apoptosis; phosphorylation

The expression by tumor cells of "intrinsic" or "acquired" resistance to chemotherapy continues to challenge progress in the treatment of cancer. While mechanisms of "intrinsic" resistance are poorly understood, there is a substantial amount of information on "acquired" resistance in tumor models [1]. In general, while the mechanism of resistance due to an alteration in a target protein involves a narrow group of drugs, overexpression of glycoprotein, PGP§ encoded by the *mdr1* gene results in tumor cell resistance to drugs of diverse structure and mechanism of action [1–3]. This phenomenon [2, 3] is also termed multidrug-resistance MDR.

A notable characteristic of cells that overexpress PGP is the cellular resistance to inhibitors of TOPO II [4, 5]. However, the mechanism of resistance to TOPO II inhibitors in cells that overexpress PGP is not due exclusively to reduced drug accumulation [5–8]. We and others have reported that alteration in DNA cleavage activity of TOPO II is potentially a more important and relevant mechanism of resistance in cells that overexpress PGP and exhibit the MDR phenotype [9, 10]. A number of pharmacological agents have been demonstrated to sensitize MDR cells [11, 12]. Our work with the calmodulin inhibitor TFP has demonstrated clearly its efficacy for TOPO II inhibitors in progressively ADR-resistant cells [7, 13]. Also, unlike other pharmacological agents, modulation of ADR sensitivity in resistant cells by calmodulin inhibitors is correlative with their potency to inhibit calmodulin-regulated processes [7]. We have also shown in MDR cells, that in contrast to

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§ *Abbreviations:* PGP, P-glycoprotein; VP-16, etoposide; ADR, Adriamycin®; TFP, trifluoperazine; KN-62, 1-[*N*,*O*-bis(1,5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-piperazine; TOPO II, topoisomerase II; and MDR, multidrug resistance.

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Vinca alkaloids, efficacy of TFP with inhibitors of TOPO II is not due to restoration of defects in drug accumulation or redistribution [7, 8]. Further, with the TOPO II inhibitors, MDR cells require less drug in the presence versus the absence of TFP for equivalent cytotoxicity [7, 8]. In the present study, we have characterized the cellular events of apoptosis, DNA cleavable complex formation, and phosphorylation of TOPO II that may be associated with the sensitization to VP-16 by inhibitors of calcium-calmodulin-dependent processes, e.g. TFP [14] and KN-62 [15]. Our results in ADR-resistant human leukemia HL-60 cells [16] demonstrate that KN-62, a selective inhibitor of calcium-calmodulin-dependent protein kinase II [15] is >4-fold more potent than TFP, and potentiation of VP-16 cytotoxicity is due to stabilizing DNA cleavable complex formation which results in apoptosis and preferential depletion of cells in S-phase.

MATERIALS AND METHODS

The parental-sensitive HL-60 (HL-60/S) cells were provided by Dr. Andrew Yen, College of Veterinary Medicine (Ithaca, NY). The ADR-resistant subline (HL-60/ADR0.05) which overexpresses the *mdr1* gene has been described previously [16]. HL-60/S and HL-60/ADR0.05 cells cultured at 37° in a humidified 5% CO_2 plus 95% air atmosphere using RPMI 1640 (M.A. Bioproducts, Gaithersburg, MD) supplemented with 2 mM L-glutamine and 10% fetal bovine serum had a doubling time of 18–20 hr.

Cytotoxicity studies were carried out using a soft-agar colony-forming assay [16]. Briefly, HL-60/S or HL-60/ADR0.05 cells were treated *in vitro* with 0.5 to 100 μM VP-16 in the absence or presence of 0.5 to 5 μM KN-62 or TFP for 1 hr at 37° in a humidified, 5% CO_2 plus 95% air atmosphere. Following treatment, cells were washed with drug-free medium and plated in 35 × 10-mm Petri dishes, and colonies from control and treated samples were counted using an automated colony counter [7, 16].

The effect of KN-62 or TFP on VP-16-induced TOPO II-mediated DNA cleavable complex formation was determined by the SDS-KCl technique [17, 18]. HL-60/S or HL-60/ADR0.05 cells were labeled with 0.02 to 0.04 μCi/mL of [^{14}C]thymidine for 24 hr and treated subsequently for 1 hr with 0.5 to 100 μM VP-16 in the absence or presence of 0.5 to 5 μM KN-62 or TFP. The drug-stimulated DNA cleavable complex was determined by the SDS-KCl technique [17, 18] and expressed as a fold-increase over the untreated control. The specific enhancement by VP-16 of TOPO II DNA cleavable complex by KN-62 or TFP was determined by the band depletion technique [18]. The HL-60/ADR0.05 cells were treated with 10–100 μM VP-16 in the absence or presence of 2 μM KN-62 or 5 μM TFP for 1 hr at 37°. Control and treated cells (2 × 10^6) were lysed in 2× Laemmli buffer [19]. Samples processed by SDS-PAGE [19] were electroblotted onto nitrocellulose [20], and TOPO II was detected with an antibody that specifically recognizes the 170 kDa (α) isoform [8]. Depletion of TOPO II due to enhanced cleavable complex formation in treated versus control cells was quantified by densitometric scanning. Since VP-16 induces apoptosis and cell cycle traverse perturbations, the effect of KN-62 and TFP on these events in HL-60/S and HL-60/ADR0.05 cells treated with VP-16 was evaluated. Cells were treated with VP-16 in the absence or presence of 2 μM KN-62 or 5 μM TFP for 1 hr at 37°, washed, and resuspended in drug-free medium. Aliquots of cells retrieved at 4 hr were fixed in 70% ethanol, stained with propidium iodide [21], and analyzed for cell cycle traverse perturbations by flow cytometry in a Becton Dickinson FACScan (Becton Dickinson, San Jose, CA). Samples retrieved at 2, 4, and 8 hr were processed for apoptosis staining in cytospin preparations with terminal transferase and fluorescein-12-dUTP (Boehringer Mannheim, Indianapolis, IN) as described earlier [22]. No less than 200 cells in at least three separate fields were counted using a fluorescence microscope to determine the percentage of apoptotic cells [22].

The effect of 2 μM KN-62 or 5 μM TFP on accumulation of VP-16 in HL-60/S and HL-60/ADR0.05 cells was determined by treating with [^{3}H]VP-16, specific activity 768 mCi/mmol (Moravek Biochemicals, Brea, CA), for 1 hr. Following treatment, the cells were centrifuged through silicone oil, digested in 0.2 N sodium hydroxide [23], and counted in a liquid scintillation counter using Ecolume (ICN Biochemicals, San Diego, CA) as the scintillation fluid. Accumulation of [^{3}H]VP-16 was expressed as nanomoles per 10^6 cells.

The resistant HL-60/ADR0.05 cells were washed and incubated in phosphate-free RPMI 1640 supplemented with 2 mM L-glutamine and 10% dialyzed fetal bovine serum for 1 hr at 37° in a humidified 5% CO_2 plus 95% air atmosphere. Cells were labeled with 160 μCi/mL of [^{32}P]orthophosphoric acid for 2 hr followed by 5 μM TFP or 2 μM KN-62 for an additional 1 hr. Cells were pelleted and lysed, and the 170 kDa TOPO II was immunoprecipitated as described earlier [8, 24]. Samples were electrophoresed on 5% SDS–polyacrylamide gels, and the signal intensity of phosphorylated TOPO II in the dried gels was determined with a Phosphorimager.

RESULTS AND DISCUSSION

The cytotoxic effects of VP-16 in the absence or presence of 2 μM KN-62 or 5 μM TFP in HL-60/S and HL-60/ADR0.05 cells are outlined in Fig. 1. Data indicate that the HL-60/ADR0.05 cells were 20- to 40-fold more resistant to VP-16 than the HL-60/S cells. In contrast to the HL-60/S cells, both KN-62 and TFP markedly enhanced cytotoxicity of VP-16 in the HL-60/ADR0.05 cells. However, KN-62 was markedly more effective than TFP in enhancing VP-16 cytotoxicity in the HL-60/ADR0.05 cells, and was able to reverse VP-16 resistance in the HL-60/ADR0.05 cells at drug concentrations that produce equivalent cytotoxicity in the HL-60/S cells.

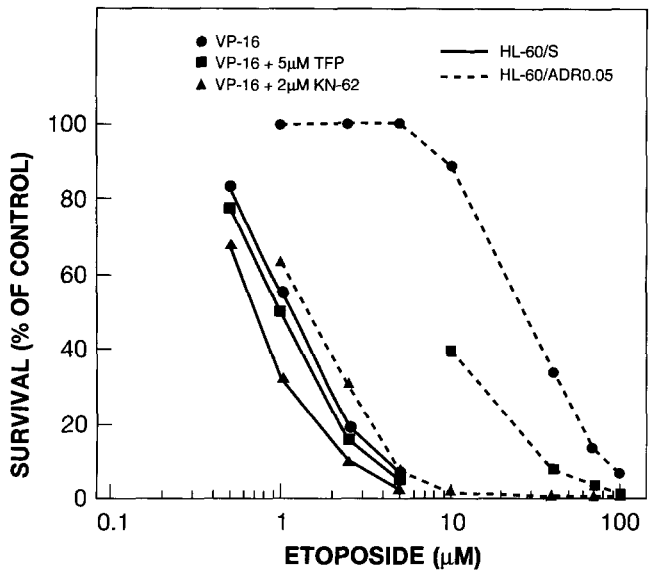

FIG. 1. Effect of 2 μM KN-62 or 5 μM TFP on the cytotoxic effects of VP-16 in HL-60/S or HL-60/ADR0.05 cells following treatment for 1 hr. Cytotoxicity was determined by a soft-agar colony assay [16]. Data are the mean values from at least triplicate experiments (SD < 15%). HL-60/S and HL-60/ADR0.05 cells were plated at a density of 1.5 × 10^4 and 4 × 10^4 cells, respectively, per 35 × 10 mm Petri dish. Colony-forming efficiency of the HL-60/S and HL-60/ADR0.05 cells was 29 and 10%, respectively.

To determine whether the effects of KN-62 and TFP on VP-16 cytotoxicity were related directly to effects on TOPO II, drug-induced stabilization of DNA cleavable complex formation and band depletion of the 170 kDa (α) TOPO II isoform was performed. Consistent with the cytotoxicity data, results in Fig. 2 demonstrated that resistance to VP-16 in the HL-60/ADR0.05 cells is due to decreased DNA cleavable complex formation, and that KN-62 is superior to TFP in enhancing VP-16-induced TOPO II-mediated DNA cleavable complex formation. The enhancement of VP-16-stabilized DNA cleavable complex formation by KN-62 or TFP is supported by the 170 kDa TOPO II band depletion data in Fig. 3. These figures also affirmed that KN-62 is more effective than TFP.

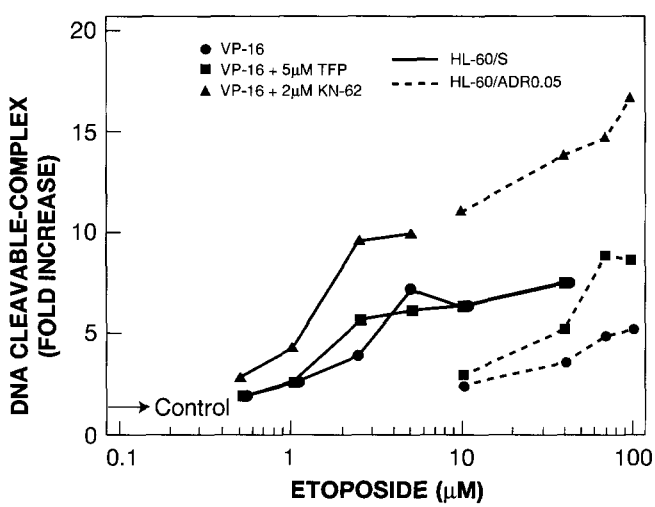

FIG. 2. Effect of 2 μM KN-62 or 5 μM TFP on VP-16-induced TOPO II-mediated DNA cleavable complex formation in HL-60/S and HL-60/ADR0.05 cells. Cells were treated for 1 hr, and DNA cleavable complex was assayed using the SDS-KCl technique [17, 18]. The standard deviation was <15% in at least triplicate experiments.

Since the induction of DNA damage by VP-16 can lead to apoptotic cell death, the effects of KN-62 and TFP on VP-16-induced cell cycle traverse perturbations and apoptosis were determined in HL-60/S and HL-60/ADR0.05 cells. Based on the data in Fig. 4, the level of apoptosis in HL-60/ADR0.05 cells is consistent with the reduced induction of DNA damage by VP-16 alone. Comparison of the DNA cleavable complex data in Fig. 2 and the apoptosis data in Fig. 4 suggests that the potentiation of the apoptotic response in cells treated with VP-16 plus TFP or KN-62 is dependent on the extent of DNA damage induced. The superiority of KN-62 to TFP was apparent in its ability to rapidly induce apoptosis when used in combination with nearly 4-fold lower concentrations of VP-16. In HL-60/S cells treated for 1 hr with 2.5 μM VP-16 in the absence or presence of 2 μM KN-62, the number of apoptotic cells was 30–40%. Similar levels of apoptosis in the HL-60/ADR0.05 cells were observed following treatment with 100 μM VP-16 alone. As shown in Fig. 5, analysis of cell cycle traverse perturbations in HL-60/ADR0.05 cells treated for 1 hr and analyzed after 4 hr in drug-free medium demonstrated that while VP-16 alone results in some accumulation of cells in the G_2 + M phase, concentrations of VP-16 plus KN-62 or TFP which induce a high level of apoptosis result in a preferential depletion of cells in S-phase. Thus, the cells depleted in S-phase could represent the apoptotic cells identified microscopically in Fig. 4.

Since agents that sensitize MDR cells enhance cellular accumulation of Vinca alkaloids, anthracyclines, or epipodophyllotoxins, the effect of KN-62 and TFP on cellular

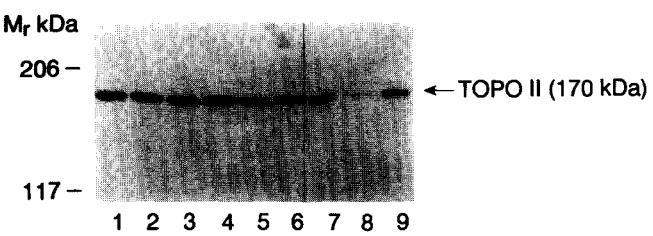

FIG. 3. Band depletion analysis of 170 kDa TOPO II in HL-60/ADR0.05 cells treated with VP-16 in the absence or presence of KN-62 or VP-16. Densitometric scanning was used to quantify the percent depletion of the 170 kDa TOPO II band in treated samples relative to control samples. Lanes 1, 2, and 3 are control, KN-62, and TFP alone, respectively; lanes 4, 5, and 6 are 10 μM VP-16 alone (15% depletion), 10 μM VP-16 + 2 μM KN-62 (46% depletion), and 10 μM VP-16 + 5 μM TFP (37% depletion), respectively; lanes 7, 8, and 9 are 100 μM VP-16 (42% depletion), 100 μM VP-16 + 2 μM KN-62 (95% depletion), and 100 μM VP-16 + 5 μM TFP (53% depletion).

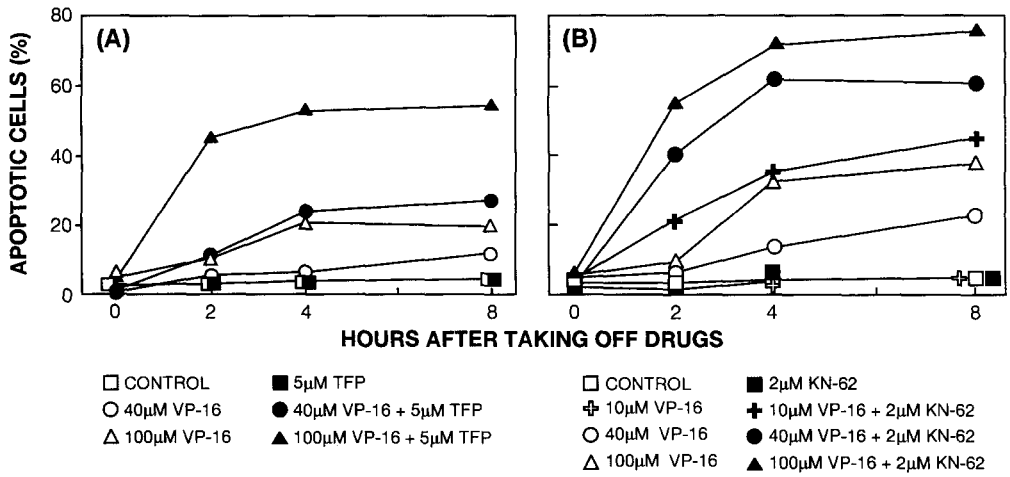

FIG. 4. Effect of a 1-hr treatment with 5 μM TFP (A) or 2 μM KN-62 (B) on VP-16-induced apoptosis in HL-60/ADR0.05 cells. Following treatment, cells were washed and resuspended in drug-free medium, and apoptosis was measured after labeling with fluorescein-12-dUTP [22]. No less than 200 cells in three separate fields were counted, and data are the mean values from at least replicate experiments (SD < 15%). Based on data in Fig. 2, the fold-increase in DNA cleavable complex in HL-60/ADR0.05 cells treated with VP-16 in the absence or presence of 5 μM TFP or 2 μM KN-62 was: 1.92, 2.58, and 3.57 at 10 μM, 40 μM, and 100 μM VP-16, respectively; 5.07 and 8.46 at 40 μM VP-16 + 5 μM TFP and 100 μM VP-16 + 5 μM TFP, respectively; 10.9, 13.65, and 16.43 at 10 μM VP-16 + 2 μM KN-62, 40 μM VP-16 + 2 μM KN-62, and 100 μM VP-16 + 2 μM KN-62, respectively.

levels of VP-16 in HL-60/S and HL-60/ADR0.05 cells was determined. Based on the results outlined in Table 1, it is apparent that for equivalent cytotoxicity the levels of VP-16 in the HL-60/ADR0.05 cells were >12-fold higher than those in the HL-60/S cells. The cellular accumulation of VP-16 was enhanced 1.5-fold in HL-60/S cells and 2-fold in HL-60/ADR0.05 cells in the presence of 2 μM KN-62. However, in the presence of 5 μM TFP, the accumulation of VP-16 was enhanced minimally in HL-60/S (<20%) cells (data not shown) and 1.5-fold in HL-60/ADR0.05 cells.

Since 170 kDa TOPO II in the HL-60/ADR0.05 cells is hypophosphorylated compared with that in the HL-60/S

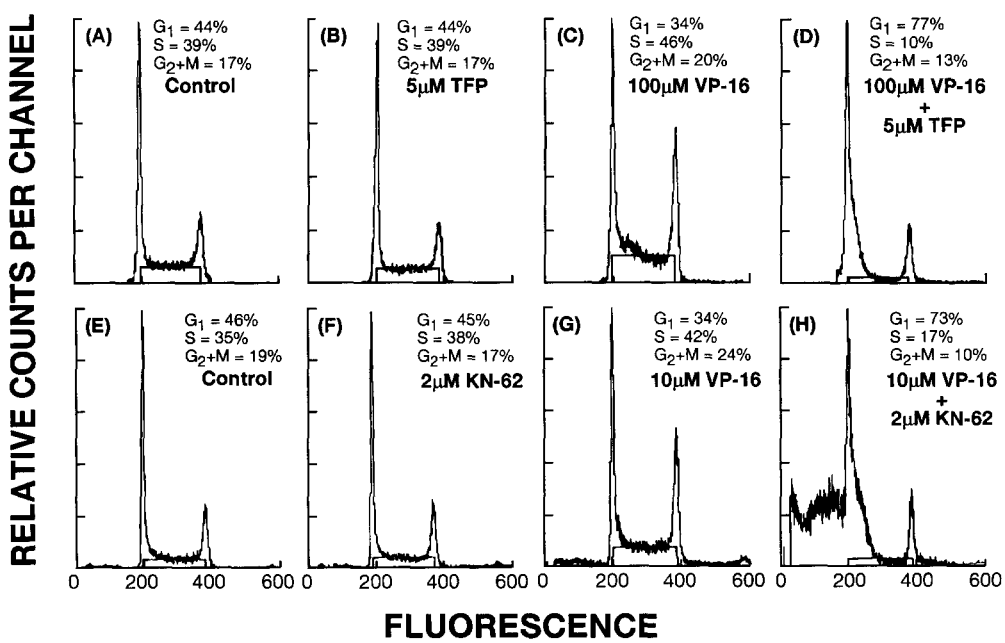

FIG. 5. Effect of a 1-hr treatment with 5 μM TFP (A–D) or 2 μM KN-62 (E–H) on VP-16-induced cell cycle traverse perturbations of HL-60/ADR0.05 cells. Following treatment, cells were washed, resuspended in drug-free medium for 4 hr, and analyzed by flow cytometry following staining with propidium iodide [21].

TABLE 1. Effect of the calcium-calmodulin inhibitors KN-62 and TFP on cellular accumulation and cytotoxicity of VP-16 in HL-60/ADR0.05 cells

	[^{3}H]VP-16 (nmol/10^6 cells)	
VP-16 (μM)*	HL-60/S	HL-60/ADR0.05
0.5	1.85† (83)‡	
0.5 + 2 μM KN-62	2.24 (68)	
1	2.73 (57)	2.63 (100)
1 + 2 μM KN-62	3.63 (32)	4.89 (63)
2.5	5.89 (18)	4.72 (100)
2.5 + 2 μM KN-62	8.17 (10)	9.6 (30)
5	9.68 (7)	7.83 (100)
5 + 2 μM KN-62	14.86 (2)	20.15 (7)
10		14.28 (86)
10 + 2 μM KN-62		36.36 (1.6)
10 + 5 μM TFP		28.55 (37)
40		47.01 (35)
40 + 2 μM KN-62		116.5 (0.1)
40 + 5 μM TFP		61.65 (8)

* Cells were treated *in vitro* with indicated extracellular concentrations of VP-16 in the absence or presence of 2 μM KN-62 and 5 μM TFP for 1 hr at 37°.

† Cellular drug levels are expressed as nanomoles [^{3}H]VP-16 per 10^6 cells. Data are the mean values from at least triplicate experiments (SD < 20%).

‡ Survival in a soft-agar colony assay expressed as a percentage of the untreated control. Data are from Fig. 1 and are the mean values from at least triplicate experiments (SD < 15%).

cells, we determined the effect of TFP and KN-62 on the phosphorylation to TOPO II. As shown in Fig. 6, the phosphorylation of 170 kDa TOPO II in the HL-60/ADR0.05 cells was enhanced 1.6- or 1.5-fold in the presence of 5 μM TFP or 2 μM KN-62, respectively.

Tumor cell resistance to inhibitors of TOPO II is often associated with the MDR phenotype, and a variety of pharmacological agents that interact with PGP have been shown to enhance cytotoxicity [11, 12]. Although the chemosensitizers do enhance drug accumulation in the resistant cells, there is no correlation between drug accumulation and cytotoxic response with TOPO II inhibitors in MDR cells [7, 8, 10]. Our studies with the calmodulin inhibitor TFP in progressively ADR-resistant L1210 mouse leukemia cells have demonstrated previously that potentiation of ADR or VP-16 cytotoxicity is not due to enhanced drug accumulation or altered drug redistribution [8, 10]. Also "reversal" of resistance was not achieved with the combination of VP-16 plus TFP. Although differences exist in the cellular accumulation of VP-16 between HL-60/S and HL-60/ADR0.05 cells, the steady-state levels of the 170 kDa TOPO II are comparable in the two cell lines [24]. It has been reported recently that KN-62 can increase accumulation of ADR in resistant ovarian carcinoma cells with the MDR phenotype [25]. The results on cellular accumulation (Table 1) suggest that for equivalent cytotoxicity the HL-60/ADR0.05 cells require > 12-fold higher levels of VP-16 than do the HL-60/S cells. The enhanced accumulation of VP-16 in the HL-60/ADR0.05 cells is not due merely to overexpression of P-glycoprotein, since a 1.5-fold increase in VP-16 levels was also observed in the HL-60/S cells that do not overexpress *mdr1* based on reverse transcriptase-polymerase chain reaction analysis [16]. While HL-60/ADR0.05 cells required less VP-16 in the presence of KN-62 or TFP to achieve equivalent cytotoxicity, cellular levels of VP-16 were >4-fold lower in the presence versus the absence of 2 μM KN-62 to achieve equivalent cell kill. The effects of both TFP and KN-62 in potentiating VP-16 cytotoxicity appear to be a consequence of the enhanced formation of VP-16-stabilized DNA cleavable complex (Figs. 2 and 3). It thus appears that given the shared inhibitory activity of TFP and KN-62 for calcium-calmodulin-regulated processes, the superiority of KN-62 over TFP as a sensitizer of VP-16 cytotoxicity may be due to its direct effects on calmodulin-dependent kinases. The superiority of KN-62 over TFP as a sensitizer of VP-16 cytotoxicity was also observed with progressively ADR-resistant L1210 mouse leukemia cells (data not shown). The activity of KN-62 in the HL-60/ADR0.05 cells is also novel, since other kinase inhibitors that are piperazine derivatives, e.g. H-7 and HA-1004, had no effects on enhancing TOPO II inhibitor cytotoxicity [26].

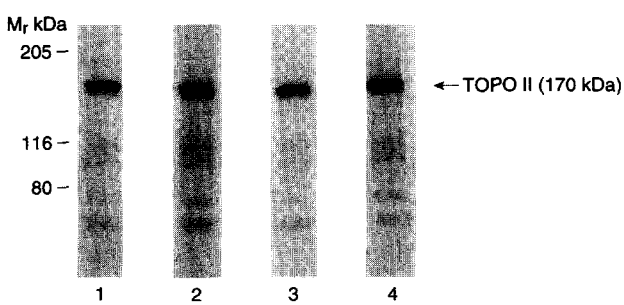

FIG. 6. Phosphorylation of TOPO II in ADR-resistant HL-60 human leukemia cells. Control (lanes 1 and 3); 5 μM TFP (lane 2); and 2 μM KN-62 (lane 4). Phosphorimager units of the TOPO II band in control (lane 1), 5 μM TFP (lane 2), control (lane 3), and 2 μM KN-62 (lane 4) were 41179, 66016, 43122, and 63836, respectively. Data from a representative experiment are shown.

The role of apoptosis as a mechanism involved in the sensitization of VP-16 cytotoxicity by KN-62 or TFP is supported by data in Figs. 4 and 5. The apoptotic response is also correlated to the magnitude of TOPO II-mediated DNA damage induced, since the differential effects of KN-62 versus TFP are VP-16 concentration dependent. The cell cycle traverse data suggest that the DNA lesions that rapidly induce apoptosis in cells treated with VP-16 plus KN-62 or TFP are restricted primarily to cells in the "S" phase.

The modest increase in the phosphorylation of 170 kDa TOPO II in the HL-60/ADR0.05 cells treated with 5 μM TFP or 2 μM KN-62 may be reconciled with the hypophosphorylation of 2–4 peptides in the HL-60/ADR0.05 cell versus HL-60/S cells [24]. Since both TFP and KN-62 inhibit kinase activity, it is possible that the increased phosphorylation of TOPO II is due to inhibition of a phosphatase. We have reported previously that okadaic acid, an inhibitor of protein phosphatases 1 and 2A, does not en-

hance the cytotoxic effects of TOPO II poisons [22]. Thus, the effects of calcium-calmodulin inhibitors TFP and KN-62 on TOPO II phosphorylation may possibly involve: (a) inhibition of protein phosphatase 2B (calcineurin) by TFP [27]; or (b) inhibition of calcium-calmodulin-dependent protein kinase II by KN-62 which prevents activation of calcineurin [28].

In summary, results from this study demonstrate that inhibitors of calcium-calmodulin-dependent processes are remarkably effective in sensitizing resistant cells to the cytotoxic effects of the TOPO II inhibitor VP-16. Both KN-62 and TFP sensitize resistant cells by enhancing VP-16-stabilized TOPO II DNA cleavable complex formation, which leads to apoptosis of cells in "S" phase of the cell cycle. Enhanced phosphorylation of TOPO II in the presence of TFP or KN-62 may be due to inhibitory effects on protein phosphatase 2B. The differential effects of KN-62 versus TFP in their ability to enhance TOPO II-mediated DNA cleavable complex formation and cytotoxicity suggest that selective inhibitory effects of KN-62 on calcium calmodulin-dependent protein kinase II may be involved. Since KN-62, unlike TFP, is able to reverse resistance to VP-16, it may be a useful agent to delineate the role of calcium-calmodulin-regulated processes in TOPO II inhibitor resistance, and potentially for sensitizing resistant cells to etoposide *in vivo*.

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