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Tumor Cell Resistance to Topoisomerase II Poisons

ROLE FOR INTRACELLULAR FREE CALCIUM IN THE SENSITIZATION BY INHIBITORS OF CALCIUM-CALMODULIN-DEPENDENT ENZYMES

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ABSTRACT. Tumor cell resistance to inhibitors of topoisomerase II (topo II) is associated frequently with the overexpression of P-glycoprotein (PGP), and strategies to overcome resistance are focused on restoring defects in drug accumulation. Inhibitors of calcium-calmodulin-dependent enzymes sensitize resistant tumor cells to the topo II poison etoposide (VP-16) by enhancing DNA damage and an apoptotic response. In the present study, we have investigated the consequences of buffering intracellular calcium with 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′*N*′-tetraacetic acid tetra(acetoxy-methyl) ester (BAPTA-AM) on the sensitizing effects of the calmodulin-dependent protein kinase II inhibitor 1-[*N*,*O*-bis(1,5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-piperazine (KN-62) in etoposide-resistant human leukemia HL-60 (HL-60/ADR0.05) cells. In cells pretreated with 20 μM BAPTA-AM for 2 hr, extracellular ATP failed to trigger intracellular calcium transients, and no effects on the accumulation of VP-16 were apparent. Also, the effect of KN-62 in significantly ($P = 0.002$ to 0.042) enhancing the accumulation of VP-16 in HL-60/ADR0.05 cells was unaffected due to pretreatment with BAPTA-AM. In contrast, pretreatment with BAPTA-AM reduced the DNA damage induced by VP-16, and significantly ($P = 0.038$) reversed the enhancement by KN-62 of VP-16-stabilized topo II-mediated DNA cleavable complex formation. The pretreatment of HL-60/ADR0.05 cells with BAPTA-AM was also associated with the hypophosphorylation of topo IIα. Consistent with the ability of BAPTA-AM to circumvent the potentiation by KN-62 of VP-16-induced DNA damage, survival of cells treated with 40 μM VP-16 in the absence of KN-62 and 10 μM VP-16 in the presence of KN-62 was significantly ($P = 0.026$ to 0.031) higher due to BAPTA-AM pretreatment. Results demonstrate that intracellular calcium transients could play a key role in the sensitization of etoposide-resistant tumor cells by inhibitors of calcium-calmodulin-dependent enzymes. BIOCHEM PHARMACOL **56**;3:345–349, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. topoisomerase II; etoposide resistance; intracellular calcium transients; calcium-calmodulin-dependent enzyme inhibitors; multidrug resistance; human leukemia cells

Tumor cell resistance to clinically useful chemotherapeutic agents [1] continues to challenge the effective management of human malignancies. Although mechanisms governing "intrinsic" resistance are poorly understood, selection *in vitro* of tumor cells adapted to grow in the presence of drug has provided information on potential mechanisms involved in the expression of "acquired" resistance [2, 3]. The overexpression of PGP‖ encoded by the *mdr1* gene can result in tumor cell resistance to drugs of diverse structure and mechanism of action [2, 3]. This phenomenon [2, 3] is also termed MDR and has been suggested to be important in clinical drug resistance.

Resistance to inhibitors of topo II [4–6] is observed frequently in tumor cells that overexpress PGP. However, reduced drug accumulation mediated by PGP does not explain the magnitude of resistance encountered with topo II inhibitors [7]. Alterations in topo II protein levels and DNA cleavage activity of topo II are thus potentially more important and possibly relevant mechanisms of resistance in cells that may overexpress PGP [4–6, 8]. A number of chemosensitizers that interact with PGP and alter the drug-sensitivity profile have been studied extensively in pre-clinical models and in patients [9, 10]. A presumed mechanism of action of these agents is restoring defects in drug accumulation [9, 10] of MDR cells. TFP an inhibitor of calmodulin [11], and KN-62, a potent inhibitor of calmodulin-dependent protein kinase II [12], sensitize tumor cells

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‖ *Abbreviations:* ADR, Adriamycin®; BAPTA-AM, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′*N*′-tetraacetic acid tetra(acetoxy-methyl) ester; KN-62, 1-[*N*,*O*-bis(1,5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-piperazine; PGP, P-glycoprotein; TFP, trifluoperazine; topo II, topoisomerase II; TPEN, *N*,*N*,*N*′*N*′-tetrakis(2-pyridylmethyl)ethylenediamine; and VP-16, etoposide.

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with the MDR phenotype to topo II poisons without restoring defects in drug accumulation. Specifically, these agents sensitize the resistant cells by enhancing drug stabilized topo II-mediated DNA cleavable complex formation and apoptosis [8, 13]. Since intracellular calcium buffers attenuate the DNA damaging and cytotoxic potential of topo II poisons [8], we investigated the effect of such treatment on the efficacy of KN-62, a potent sensitizer of etoposide-induced DNA damage and cytotoxicity in MDR cells [13]. The results demonstrated that in etoposide-resistant cells, the intracellular calcium buffer BAPTA-AM does not affect cellular drug accumulation in the absence or presence of KN-62. However, pretreatment with BAPTA-AM significantly inhibited the potentiation by KN-62 of etoposide-stabilized topo II-mediated DNA cleavable complex formation and cytotoxicity, suggesting a requirement for intracellular calcium transients. Further, the effects of the intracellular calcium buffer are also possibly mediated by the hypophosphorylation of topo IIα.

MATERIALS AND METHODS

The parental-sensitive HL-60 (HL-60/S) cells were provided by Dr. Andrew Yen, College of Veterinary Medicine, Cornell University. The ADR-resistant subline (HL-60/ADR0.05), which overexpresses the *mdr1* gene, was isolated as previously described [14]. The HL-60/ADR0.05 cells were cultured at 37° in a humidified 5% CO_2 plus 95% air atmosphere using RPMI 1640 (M.A. Bioproducts) supplemented with 2 mM L-glutamine and 10% fetal bovine serum and had a doubling time of 18–20 hr.

The intracellular calcium buffer BAPTA-AM and TPEN were obtained from Calbiochem-Novabiochem International. Log phase cultures of HL-60/ADR0.05 cells were treated with BAPTA-AM or TPEN to determine the effects on intracellular calcium transients, phosphorylation of topo IIα, and the sensitizing effects of KN-62 on etoposide-stabilized DNA cleavable complex formation and cytotoxicity.

Calcium transients induced by extracellular ATP were determined as described by Dubyak *et al.* [15]. Briefly, HL-60/ADR0.05 cells in suspension and pretreated with 20 μM BAPTA-AM or TPEN were loaded with fura2-AM ester (Molecular Probes) for 30–40 min. Following centrifugation and removal of the supernatant, the cells were suspended in ice-cold basal salt solution prior to measurements of ATP-induced calcium transients. All measurements were carried out at 37° using a stirred quartz cuvette.

Cytotoxicity studies were carried out using a soft-agar colony forming assay [8, 13]. Briefly, the HL-60/ADR0.05 cells were pretreated *in vitro* with BAPTA-AM or TPEN followed by 10–40 μM VP-16 in the absence or presence of 2 μM KN-62 for 1 hr at 37° in a humidified, 5% CO_2 plus 95% air atmosphere. Following treatment, cells were washed, reincubated for 24 hr in drug-free medium, and plated in 35 × 10-mm Petri dishes. Colonies from control and treated samples were counted using an automated colony counter [8, 13].

The effect of pretreatment with BAPTA-AM on sensitization by KN-62 of VP-16 stabilized topo II-mediated DNA cleavable complex formation was determined by the SDS-KCl technique [8, 16, 17]. The HL-60/ADR0.05 cells were labeled with 0.02 to 0.04 μCi/mL of [^{14}C]thymidine for 24 hr, pretreated with 20 μM BAPTA-AM, and subsequently treated for 1 hr with 10–40 μM VP-16 in the absence or presence of 2 μM KN-62. The drug-stimulated DNA cleavable complex was determined by the SDS-KCl technique [8] and expressed as a fold-increase over the untreated control.

The effect of 2 μM KN-62 on accumulation of VP-16 in HL-60/ADR0.05 cells treated with or without BAPTA-AM was determined by exposing the cells to [^{3}H]VP-16, specific activity 768 mCi/mmol (Moravek Biochemicals) for 1 hr. Following treatment, the cells were centrifuged through silicone oil, digested in 0.2 N sodium hydroxide [8, 18], and counted in a liquid scintillation counter using Ecolume (ICN Biochemicals) as the scintillation fluid. Accumulation of [^{3}H]VP-16 was expressed as nanomoles per 10^6 cells.

The effect of pretreatment with BAPTA-AM on the phosphorylation of topo IIα was determined in cells metabolically labeled with [^{32}P]orthophosphoric acid and immunoprecipitating the α isoform of topo II. The 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 in the absence or presence of 20 μM BAPTA-AM for 1 hr at 37° in a humidified 5% CO_2 plus 95% air atmosphere. Cells were subsequently labeled with 160 μCi/mL of [^{32}P]orthophosphoric acid for 2 hr. Cells were pelleted and lysed, and the 170 kDa (α isoform) topo II protein was immunoprecipitated as described earlier [8, 13]. 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 [8, 13].

RESULTS AND DISCUSSION

Intracellular calcium transients in the HL-60/ADR0.05 cells following exposure to BAPTA-AM was determined fluorimetrically [15]. HL-60 cells express G-protein coupled $P2Y_2$ nucleotide receptors that activate IP_3 accumulation and Ca^{2+} mobilization [15]. Thus, intracellular calcium transients due to mobilization were elicited by the addition of 100 μM extracellular ATP [15]. The characteristic intracellular calcium transients triggered by ATP in HL-60/ADR0.05 cells are shown in Fig. 1. In contrast, in HL-60 cells pretreated with 20 μM BAPTA-AM for 2 hr, ATP did not trigger intracellular calcium transients.

We have reported previously that KN-62 [12] is a potent sensitizer of HL-60/ADR0.05 cells and can enhance cellular accumulation of VP-16 [13]. To determine whether BAPTA-AM affects PGP function or the sensitizing effect

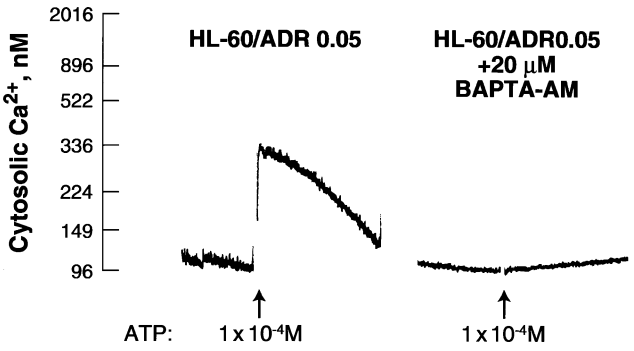

FIG. 1. Inhibition of ATP-induced Ca^{2+} transients in HL-60/ADR0.05 cells treated with BAPTA-AM. The cells pretreated with 20 μM BAPTA-AM for 2 hr were loaded with fura2, and Ca^{2+} was measured [15] as described in Materials and Methods. The data presented on Ca^{2+} transients are from a representative experiment using a single cell preparation, and were qualitatively and quantitatively similar to results from at least three independent experiments.

of KN-62, we determined the cellular accumulation of [^{3}H]VP-16. The results from these experiments shown in Fig. 2 demonstrate that pretreatment with BAPTA-AM did not alter significantly (P = 0.42 to 0.98) the cellular accumulation of VP-16. Further, while KN-62 significantly (P = 0.002 to 0.042) enhanced the accumulation of VP-16, the effects of KN-62 in increasing cellular accumulation of VP-16 were not significantly (P = 0.66 to 0.98) affected by the pretreatment with BAPTA-AM.

Since inhibitors of calcium-calmodulin-dependent enzymes enhance the drug-stabilized topo II-mediated DNA cleavable complex formation, the effects of pretreatment with BAPTA-AM on the modulation of VP-16-induced DNA cleavage by KN-62 were determined. The results on topo II-mediated DNA cleavable complex formation with VP-16 in BAPTA-AM and/or KN-62 treated cells are outlined in Fig. 3. The data suggest that pretreatment with BAPTA-AM significantly reduced the DNA cleavage induced by VP-16 (P = 0.015 to 0.037). Also, while KN-62

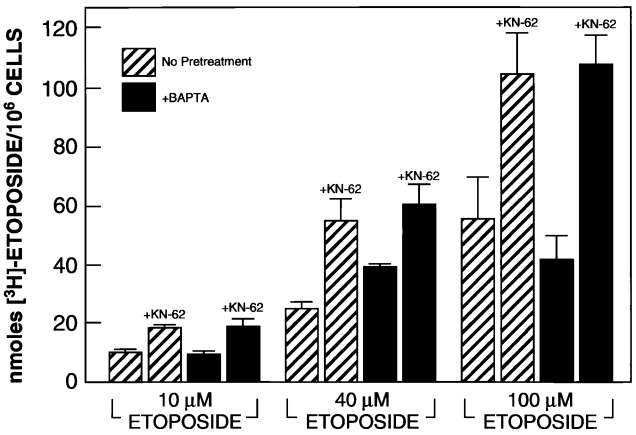

FIG. 2. Effect of pretreatment with 20 μM BAPTA-AM on the accumulation of [^{3}H]VP-16 in the absence or presence of 2 μM KN-62. Results are the means (± SEM) from at least triplicate experiments.

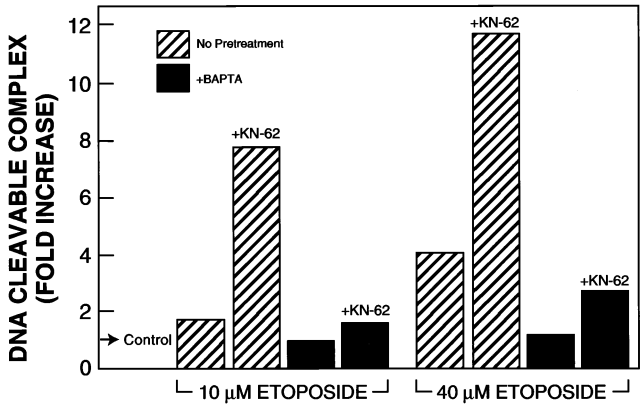

FIG. 3. Effect of BAPTA-AM on stimulation by KN-62 of VP-16-stabilized topo II-mediated DNA cleavable complex formation. Data are the mean values from replicate experiments; range, 20%.

can enhance DNA cleavage induced by VP-16, this potentiation was reduced significantly (P = 0.038) in the HL-60/ADR0.05 cells pretreated with BAPTA-AM.

We have reported previously that BAPTA-AM can induce hypophosphorylation of topo IIα, and this results in decreased drug-stimulated DNA cleavage [8]. We have determined the effects of BAPTA-AM on the phosphorylation state of topo IIα in HL-60/ADR0.05 cells, and the results are shown in Fig. 4. The data suggest that inhibiting intracellular calcium transients by BAPTA-AM in HL-60/ADR0.05 cells can result in the hypophosphorylation of topo IIα.

Since pretreatment with BAPTA-AM significantly affected the DNA cleavage induced by VP-16 and its modulation by KN-62, we investigated the outcome of such treatment on cell survival, using the soft-agar colony assay. The results from these experiments are shown in Fig. 5. The data suggest the following: 1) the cytotoxic effects of VP-16 are reduced in cells pretreated with BAPTA-AM but not with TPEN, an agent that does not interfere with intracellular calcium transients [19]; and 2) while KN-62 can significantly enhance the cytotoxic effects of VP-16 (P =

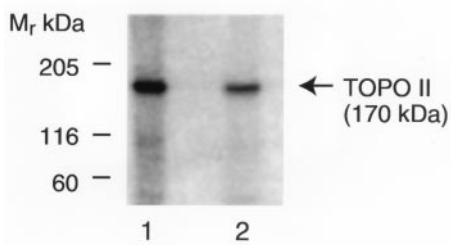

FIG. 4. Phosphorylated topo IIα in BAPTA-AM-treated HL-60/ADR0.05 cells. Cells either untreated (lane 1) or treated with 20 μM BAPTA-AM (lane 2) for 1 hr were metabolically labeled with [^{32}P]orthophosphoric acid. Nuclei were isolated from 4 × 10^6 cells, lysed in buffer [8], immunoprecipitated with antiserum specific for topo IIα, and resolved by SDS-PAGE for autoradiography. Based on PhosphorImager or densitometric analysis, the level of phosphorylated topo IIα in untreated cells was 1.8-fold higher than in BAPTA-AM-treated cells.

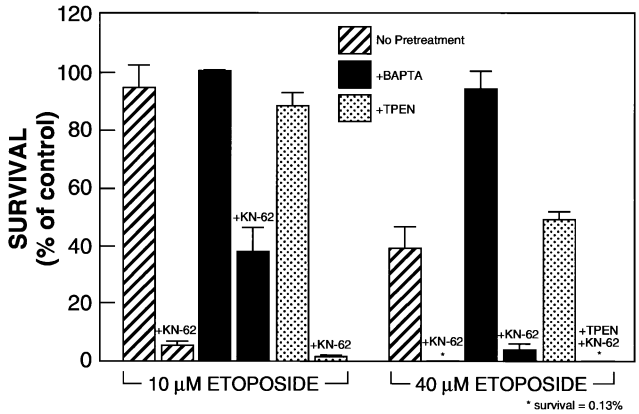

FIG. 5. Survival in a soft-agar colony assay of HL-60/ADR0.05 cells pretreated with 20 μM BAPTA-AM or TPEN for 2 hr followed by VP-16 ± 2 μM KN-62 for an additional hour. Control (untreated) Petri dishes contained 4 × 10^4 cells, and the BAPTA-AM- or TPEN-treated Petri dishes contained 8 × 10^4 cells. Colony forming efficiency in the control or cells treated with BAPTA-AM or TPEN alone was 25–30%. Bars, standard error; N = 3.

0.001 to 0.003), its potency is significantly overcome in cells pretreated with BAPTA-AM ($P = 0.031$) but not with TPEN ($P = 0.2$ to 0.52).

Drug resistance to inhibitors of topo II can occur in tumor cells in the absence or presence of PGP overexpression [2–6]. Mechanisms usually associated with resistance to topo II inhibitors include defective drug accumulation, reduced topo II enzyme levels, or a mutant topo II enzyme [5, 6]. Because defective drug accumulation mediated by PGP is considered a key mechanism of resistance to topo II poisons [2, 3], a number of clinical protocols to modulate such resistance are focused on agents that interfere with PGP function, with the goal of restoring defective drug accumulation [9, 10]. We have reported previously that inhibitors of calcium-calmodulin-regulated processes sensitize resistant cells by mechanisms that do not rely on restoration of reduced drug accumulation [7, 13]. Also, in the presence of the calmodulin inhibitor, markedly lower concentrations of ADR or VP-16 are required to produce equivalent cell kill [7, 13]. The inhibitors of calcium-calmodulin-regulated processes sensitize the resistant cells to topo II poisons by stimulating drug-stabilized DNA cleavable complex formation and, consequently, the apoptotic response [13].

The results from this study suggest that sensitizing HL-60/ADR0.05 cells to the cellular effects of VP-16 by KN-62 can be reversed significantly in cells pretreated with BAPTA-AM. These effects are possibly directly related to buffering of intracellular calcium, because pretreatment with TPEN [19], which does not affect ATP-stimulated calcium transients (data not shown), did not alter significantly the sensitizing effects of KN-62. The data on cellular accumulation of VP-16 suggest that KN-62 can increase drug levels significantly. It is noteworthy that this effect of KN-62 was not altered in BAPTA-AM-treated cells, suggesting that mechanisms of increasing drug accumulation due to interaction with PGP are not involved. The data also demonstrate that the sensitizing effects of KN-62 are not merely related to increased drug accumulation.

It is generally accepted that agents which sensitize tumor cells with the MDR phenotype exert their effects by augmenting drug accumulation [9, 10]. While KN-62 can increase cellular accumulation of VP-16, the decreased DNA damage due to BAPTA-AM pretreatment is due to mechanisms unrelated to the lowering of cellular VP-16 levels. Also, the effects of BAPTA-AM are quite remarkable on negating the sensitization of DNA damage by KN-62. The data on phosphorylation of topo IIα suggest that the effects of BAPTA-AM may also be due to the hypophosphorylation of the enzyme. These results are similar to the hypophosphorylation of topo IIα observed in wild-type cells treated with BAPTA-AM [8]. Further, since KN-62 can induce the hyperphosphorylation of topo IIα in the HL-60/ADR0.05 cells [13], the reversal of its effects due to BAPTA-AM pretreatment may be linked to effects on the phosphorylation state of the enzyme.

The correlation of DNA damage induced by topo II poisons and the cytotoxic response has not been firmly established. However, in the HL-60/ADR0.05 cells, the sensitization by KN-62 of VP-16-induced DNA damage is correlative with induction of apoptosis and cell survival [13]. The present findings on pretreatment with BAPTA-AM suggest that reversal of the sensitization by KN-62 of VP-16-induced DNA damage also results in significantly enhanced survival. Although pretreatment with TPEN can also reduce VP-16-induced DNA damage (data not presented), unlike BAPTA the pretreatment with TPEN does not affect cell survival. While circumvention of the effects of KN-62 on etoposide cytotoxicity are not apparent, it is possible that a reversal of the cellular effects of BAPTA may occur more rapidly, allowing for some sensitization by KN-62. Also, the effects of KN-62 in enhancing etoposide cytotoxicity in the HL-60/ADR0.05 cells may involve other mechanisms, including the stabilization of DNA cleavable complex formation.

In summary, the present results demonstrate that buffering intracellular calcium with BAPTA-AM can reverse the sensitizing effects of KN-62 in topo II poison resistant human leukemia HL-60 cells. The results on the effect of BAPTA and/or KN-62 on topo IIα and the cellular response with etoposide in HL-60/ADR0.05 cells are summarized in Table 1. The effects of BAPTA-AM treatment are not unique to KN-62, since similar results were also obtained with the calmodulin inhibitor TFP (data not presented), which is significantly less effective than KN-62 in sensitizing HL-60/ADR0.05 cells. Because calmodulin inhibitors require the presence of intracellular free calcium for their binding to the target proteins [11] and exerting a biological effect, the buffering of intracellular calcium with BAPTA-AM may be inhibiting the sensitizing effect of TFP by affecting the binding. Similarly, with KN-62 it is possible that calcium-dependent binding responsible for

TABLE 1. Effects of KN-62 in HL-60/ADR0.05 cells treated without and with BAPTA-AM

Effect of KN-62 on:	Without BAPTA pretreatment	With BAPTA pretreatment
Cellular accumulation of etoposide	↑	↑
Etoposide-stabilized DNA cleavable complex formation	↑	↓
Etoposide cytotoxicity	↑	↓
Phosphorylation of topoisomerase IIα	↑ [13]	Not determined

biological activity may be impaired. Alternatively, since KN-62 and TFP can induce hyperphosphorylation of topo IIα [13], the hypophosphorylation of topo IIα induced with BAPTA-AM may be responsible for abrogating the sensitizing effect of these agents. Thus, addressing the role of calcium-calmodulin-dependent processes in drug-stabilized topo II-mediated DNA cleavable complex formation could provide important new information on the mechanisms of action and resistance to topo II poisons.

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