Attenuation of drug-stimulated topoisomerase II–DNA cleavable complex formation in wild-type HL-60 cells treated with an intracellular calcium buffer is correlated with decreased cytotoxicity and site-specific hypophosphorylation of topoisomerase IIα

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Topoisomerase II (topo II), an essential enzyme for cell viability, is also the target for clinically important anti-neoplastic agents that stimulate topo II-mediated DNA scission. The role of alterations in topo IIα phosphorylation and its effect on drug-induced DNA damage and cytotoxicity were investigated. Following loading of HL-60 cells with the calcium buffer 1,2-bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid tetra(acetoxymethyl) ester (BAPTA-AM), which abrogates intracellular Ca^{2+} transients, a significant decrease in etoposide (VP-16)- or amsacrine (m-AMSA)-stabilized topo II–DNA cleavable complex formation and a corresponding decrease in cytotoxicity was observed. In a cell-free system, nuclear extracts from BAPTA-AM-treated cells exhibited markedly less activity when assayed for VP-16-stabilized topo II–DNA complex formation, but not decatenation of kinetoplast DNA. In contrast, the loading of HL-60 cells with *N*,*N*,*N*′,*N*′-tetrakis-(2-pyridyl)ethylenediamine (TPEN), which binds heavy metals without disturbing calcium or magnesium concentrations, did not significantly affect VP-16-stimulated topo II–DNA cleavable complex formation or cytotoxicity. In HL-60 cells the accumulation of BAPTA, but not TPEN, also led to the hypophosphorylation of topo IIα. Tryptic phosphopeptide mapping of topo IIα protein from HL-60 cells revealed: (a) eight major phosphorylation sites in untreated cells; (b) hypophosphorylation of two out of eight sites in BAPTA-AM-treated cells; and (c) hypophosphorylation of between two and four out of eight sites in topo II-poison-resistant HL-60 cells. The two hypophosphorylated sites present following BAPTA-AM treatment of wild-type cells were identical with the hypophosphorylated sites in the resistant cells, but were not the same as the sites that are substrates for casein kinase II [Wells, Addison, Fry, Ganapathi and Hickson (1994) J. Biol. Chem. **269**, 29746–29751]. In summary, changes in intracellular Ca^{2+} transients that lead to the site-specific hypophosphorylation of topo IIα are possibly involved in regulating the DNA damage caused by and the cytotoxic potential of topo II poisons.

INTRODUCTION

Topoisomerases alter DNA topology for the efficient processing of genetic material [1,2]. The two well characterized topoisomerases, topoisomerase I and topoisomerase II (topo II), which are essential for DNA metabolism, are also the targets for clinically effective anti-tumour agents [3], e.g. the camptothecins (topotecan, irinotecan), the anthracyclines (daunorubicin, doxorubicin) and the epipodophyllotoxins [VP-16 (etoposide) and teniposide]. The intrinsic sensitivity of tumour cells to topo II poisons has been suggested to be correlated with topo II enzyme levels or defects in drug accumulation [3]. The post-translational modification of topo IIα by phosphorylation regulates its activity, and site-specific phosphorylation during cell cycle transit has been reported [4,5]. Although topo IIα is hypophosphorylated in resistant tumour cells in the absence or presence of *mdr1* overexpression [6–8], the role of the phosphorylation state of topo II in governing drug-stimulated DNA scission, as well as the correlation between DNA damage and the cytotoxic response, is controversial. The functional significance of hypophosphorylated topo II in drug-resistant cells and defining a role for the cellular phosphorylation of topo II are also complicated by an inability to identify specific cellular events that can be manipulated to affect the intrinsic sensitivity of topo IIα to agents that poison the enzyme.

We have reported previously a decrease in the formation of VP-16-stabilized topo II–DNA-cleavable complexes and altered phosphorylation of topo IIα caused by buffering of intracellular Ca^{2+} in wild-type HL-60 cells, and the site-specific hypophosphorylation of topo IIα in doxorubicin-resistant human leukaemia HL-60 cells [8]. The cell-permeant intracellular calcium buffer BAPTA-AM [1,2-bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid tetra(acetoxymethyl) ester] and the chelator TPEN [*N*,*N*,*N*′,*N*′-tetrakis-(2-pyridyl)ethylenediamine], which does not disturb calcium or magnesium concentrations, are useful agents for providing insights into the role of free cytosolic Ca^{2+} [9,10]. Thus we sought to test the hypothesis that intracellular free Ca^{2+} transients and the site-specific phosphory-

Abbreviations used: m-AMSA, amsacrine; VP-16, etoposide; topo II, topoisomerase II; BAPTA-AM, 1,2-bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid tetra(acetoxymethyl) ester; TPEN, *N*,*N*,*N*′,*N*′-tetrakis-(2-pyridyl)ethylenediamine; kDNA, kinetoplast DNA; SV40, simian virus 40.

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lation of topo IIα are required for manifestation of topo II-mediated DNA scission and cytotoxicity by agents such as m-AMSA (amsacrine), doxorubicin and VP-16. In the present study, using wild-type human leukaemia HL-60 (HL-60/S) cells, we have investigated the role of intracellular Ca^{2+} transients and of the phosphorylation of topo II on the DNA-damaging and cytotoxic effects of drugs that poison this enzyme. We show that, in wild-type HL-60 cells, the buffering of intracellular free Ca^{2+} results in: (a) overall and site-specific hypophosphorylation of the topo IIα protein; and (b) attenuated DNA damage and a corresponding decrease in the cytotoxic effects of m-AMSA, doxorubicin or VP-16 which mimics the phenotype of tumour cells that are resistant to topo II poisons.

MATERIALS AND METHODS

Wild-type HL-60 (HL-60/S) cells were obtained from Dr. Andrew Yen (College of Veterinary Medicine, Cornell University, Ithaca, NY, U.S.A.). Cultures of HL-60/S cells were maintained in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum and 2 mM L-glutamine (Biowhittaker, Gaithersburg, MD, U.S.A.) at 37 °C in a humidified 5% CO_2/95% air atmosphere. The resistant subline of HL-60 cells developed by culturing the wild-type cells in increasing concentrations of doxorubicin (0.025–0.05 μg/ml) has been described previously [8]. The doxorubicin-resistant subline (HL-60/DOX0.05) was maintained in the absence of doxorubicin during experimentation. The doubling time *in vitro* of the HL-60 and HL-60/DOX0.05 cells was 18–20 h.

Exponential-phase cultures of HL-60 cells were treated for 1–2 h with 10–20 μM BAPTA-AM or TPEN (Calbiochem–Novabiochem International, San Diego, CA, U.S.A.) to determine the effect on intracellular Ca^{2+} transients and as a pretreatment for evaluating effects on drug-stimulated topo II–DNA cleavable complex formation, phosphorylation of topo IIα and the cytotoxic response.

ATP-induced Ca^{2+} transients were determined as described by Dubyak et al. [11]. Briefly, HL-60 cells in suspension pretreated with BAPTA-AM or TPEN were loaded with fura2-AM (Molecular Probes, Eugene, OR, U.S.A.) for 30–40 min. Following centrifugation (200 ***g***; 10 min) and removal of the supernatant, the cells were suspended in ice-cold basal salt solution prior to the measurement of ATP-induced Ca^{2+} transients. All measurements were carried out at 37 °C using a stirred quartz cuvette.

The effects of VP-16 and m-AMSA on the induction of topo II-mediated DNA scission was determined by measuring the precipitation of protein–DNA complexes by a modification of the SDS/KCl technique described by Zwelling et al. [12]. Cells were labelled for 24 h with 0.02–0.04 μCi/ml [^{14}C]thymidine (specific radioactivity 53 mCi/mmol; Amersham, Arlington Heights, IL, U.S.A.). The labelled HL-60 cells were pretreated with BAPTA-AM or TPEN, followed by treatment with 0.5–100 μM VP-16 or m-AMSA for 1 h, and were assayed for protein–DNA complex formation as described previously [8].

Cytotoxicity studies *in vitro* were carried out using a soft-agar colony assay [8] following pretreatment with BAPTA-AM or TPEN and subsequent exposure to m-AMSA, doxorubicin or VP-16 for 1 h. The colony-forming efficiency of the HL-60 cells under these conditions was 29%.

Nuclei were isolated for the preparation of nuclear extracts as described previously [13]. The amounts of topo IIα protein in nuclear extracts containing equivalent amounts of protein were determined by immunoblotting [8]. The decatenating activity [14] of topo II in nuclear extracts from control and BAPTA-AM-treated cells was determined using kinetoplast DNA (kDNA) (Topogen, Columbus, OH, U.S.A.) as substrate. The drug-stimulated formation of covalent topo II–DNA complexes by nuclear extracts from control or BAPTA-AM-treated cells was determined by the precipitation of 3′-end-^{32}P-labelled simian virus 40 (SV40) DNA in a cell-free system, as described by Zwelling et al. [12]. Briefly, SV40 DNA Form I was linearized with *Eco*RI and 3′-end-labelled with [^{32}P]dATP. The 3′-end-^{32}P-labelled SV40 DNA was incubated with either 0.1 μg or 1.0 μg of nuclear extract from control or BAPTA-AM-treated cells in the presence of 10–100 μM VP-16 for 30 min at 37 °C [12]. No BAPTA was included in the reaction for measuring VP-16-stimulated protein–DNA complex formation with the 3′-end-^{32}P-labelled SV40 DNA. The reaction was stopped by the addition of SDS, and the protein–DNA complexes were precipitated by the addition of by KCl [12]. The precipitate was washed twice with 100 mM KCl, dissolved in water at 65 °C [12] and added to Ecolume (ICN Pharmaceutical, Costa Mesa, CA, U.S.A.), and radioactivity was counted in a liquid scintillation counter.

Phosphorylation of topo IIα in control, BAPTA-AM- or TPEN-pretreated HL-60 cells was determined by metabolic labelling with ^{32}P-labelled P_i [6–8]. Nuclei were isolated from the labelled cells and lysed in RIPA buffer [6–8], and the topo IIα in lysates containing equivalent numbers of cells or similar amounts of protein was immunoprecipitated using a polyclonal antibody that recognizes the 170 kDa (α) protein [6–8]. Details of the technique for metabolic labelling and immunoprecipitation have been reported previously [6–8]. Levels of phosphorylated topo IIα protein were determined by densitometric scanning of autoradiograms or by the use of a Phosphorimager.

Phosphopeptide analysis of the immunoprecipitated 170 kDa (α) topo II was carried out as described by Boyle et al. [15] and Wells et al. [16]. Briefly, the band corresponding to the 170 kDa (α) topo II protein was excised from the dried, unfixed gel and eluted with 50 mM ammonium bicarbonate/0.1% SDS/0.5% 2-mercaptoethanol overnight. The protein was precipitated with 100% (w/v) trichloroacetic acid and oxidized with performic acid. Protein samples were digested overnight in Tos-Phe-CH_2Cl ('TPCK')-treated trypsin, and the radioactivity was determined by Čerenkov counting. Aliquots of the phosphopeptides (containing equivalent d.p.m.) reconstituted in pH 1.9 electrophoresis buffer were loaded on to thin-layer cellulose plates and analysed by electrophoresis with pH 1.9 buffer in the horizontal dimension, and with phospho-chromatography buffer in the vertical dimension [15,16].

RESULTS

Intracellular Ca^{2+} transients in HL-60 cells following exposure to BAPTA-AM or TPEN were determined fluorimetrically [11]. HL-60 cells express G-protein-coupled P_{2Y2} nucleotide receptors that activate InsP_3 accumulation and Ca^{2+} mobilization [11]. Thus intracellular Ca^{2+} transients due to mobilization were elicited by the extracellular addition of 100 μM ATP [11]. The characteristic intracellular Ca^{2+} transients triggered by ATP in control HL-60 cells are shown in Figure 1. In contrast, in HL-60 cells pretreated with 20 μM BAPTA-AM for 1 h or with 10 μM BAPTA-AM for 2 h, ATP did not trigger intracellular Ca^{2+} transients. However, the intracellular Ca^{2+} transients induced by ATP in HL-60 cells pretreated with TPEN were comparable with those in control cells.

Based on the differential repression of ATP-stimulated intracellular Ca^{2+} transients, the effects of pretreatment with BAPTA-AM or TPEN on the stimulation of topo II–DNA cleavable complex formation by non-intercalating (e.g. VP-16)

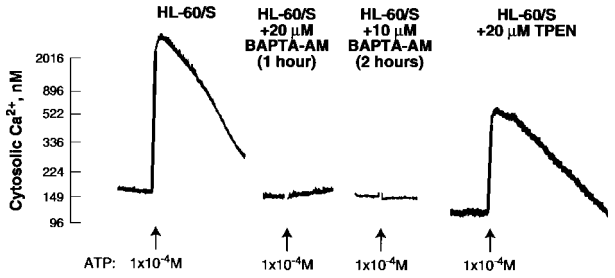

Figure 1 Inhibition of ATP-induced Ca^{2+} transients in HL-60 cells treated with BAPTA-AM, but not in those treated with TPEN

HL-60/S cells pretreated with indicated concentrations of BAPTA-AM or TPEN for 1–2 h were loaded with fura2 and Ca^{2+} was measured [11] as described in the Materials and methods section. The data presented are from a representative experiment using a single cell preparation, and are qualitatively and quantitatively similar to results from at least three independent experiments.

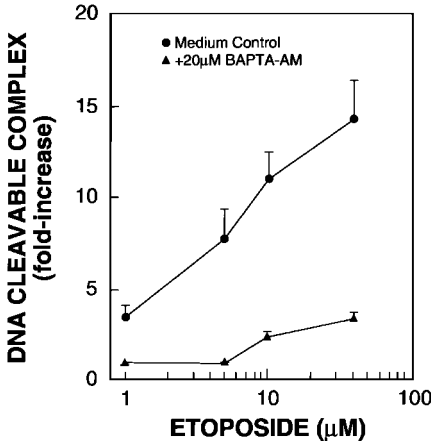

Figure 2 Effect of BAPTA-AM on stimulation by VP-16 (etoposide) of topo II–DNA cleavable complex formation

HL-60 cells labelled with [^{14}C]thymidine were pretreated for 2 h with 20 μM BAPTA-AM, followed by treatment with VP-16 (etoposide) for 1 h. SDS/KCl precipitation and analysis of the protein–DNA complex was carried out as described in the Materials and methods section. The fold increase is the ratio of the amounts of precipitated [^{14}C]DNA–protein complex with and without etoposide treatment. Bars indicate S.E.M. Statistical analysis (Student's *t* test) indicated that the *P* values for the differences between control and BAPTA-AM-treated cells at 1, 5, 10 and 40 μM VP-16 were 0.001, 0.004, 0.04 and 0.021 respectively.

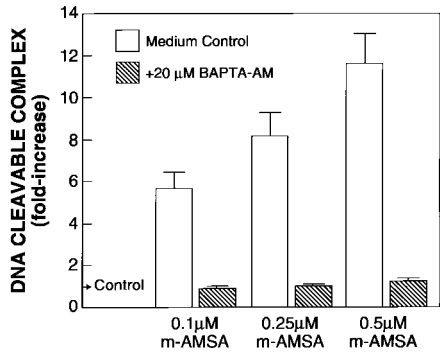

Figure 3 Effect of BAPTA-AM on stimulation by m-AMSA of topo II–DNA cleavable complex formation

HL-60 cells labelled with [^{14}C]thymidine were pretreated for 2 h with 20 μM BAPTA-AM, followed by treatment with m-AMSA for 1 h. SDS/KCl precipitation and analysis of the protein–DNA complex was carried out as described in the Materials and methods section. The fold increase is the ratio of the amounts of precipitated [^{14}C]DNA–protein complex with and without m-AMSA treatment. The control is the normalized (1-fold) increase without drug treatment. Bars indicate S.E.M.

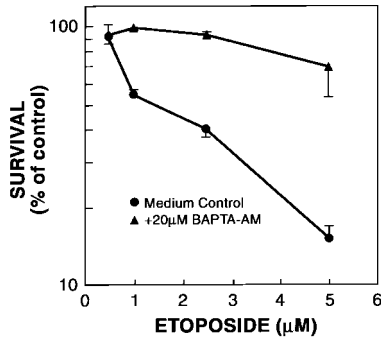

Figure 4 Survival in a soft-agar colony assay of HL-60 cells pretreated with 20 μM BAPTA-AM for 2 h followed by VP-16 (etoposide) treatment for an additional 1 h

The colony-forming efficiency in control cells or cells treated with BAPTA-AM alone was 25–30%. Bars indicate S.E.M.

and intercalating (e.g. m-AMSA) topo II poisons was determined. The results from these experiments with VP-16 and m-AMSA are outlined in Figures 2 and 3 respectively. As expected, in untreated cells VP-16 produced a dose-dependent increase in topo II–DNA cleavable complex formation (Figure 2). This response to VP-16 in stimulating topo II–DNA cleavable complex formation was significantly attenuated in cells pretreated with BAPTA-AM (Figure 2). In contrast, topo II–DNA cleavable complex formation in the presence of VP-16 in cells pretreated with TPEN was not significantly different from that in the untreated control (results not shown). Also, the effects of BAPTA-AM in attenuating DNA damage are not unique to VP-16, since the formation of covalent topo II–DNA complexes induced by m-AMSA was also significantly decreased in BAPTA-AM-treated cells (Figure 3).

Since pretreatment with BAPTA-AM affected DNA damage induced by VP-16 or m-AMSA, the relationship with cell survival was evaluated using a soft-agar colony assay. The data in Figure 4 demonstrate that a dose-dependent decrease in cell survival was apparent in VP-16-treated control cells. In contrast, in the cells pretreated with BAPTA-AM, there was a minimal effect on cell survival over the wide range of VP-16 concentrations tested. Similarly, as shown in Figure 5, pretreatment with BAPTA-AM significantly decreased cell death following m-AMSA or doxorubicin treatment. Pretreatment with TPEN (which does not repress ATP-induced Ca^{2+} transients) followed by VP-16, m-AMSA or doxorubicin produced dose–response survival curves similar to those of the control cells (results not shown). Overall, the results demonstrate that abrogation of intracellular Ca^{2+} transients with BAPTA-AM significantly decreases DNA damage induced by, and the subsequent cytotoxic effects of, the topo II poisons VP-16, m-AMSA and doxorubicin. Since the effects on ATP-stimulated intracellular Ca^{2+} transients in BAPTA-AM-treated cells were dose- and time-dependent, similar levels of attenuation of the stimulation of topo II-mediated DNA scission or cytotoxicity by topo II poisons were also obtained

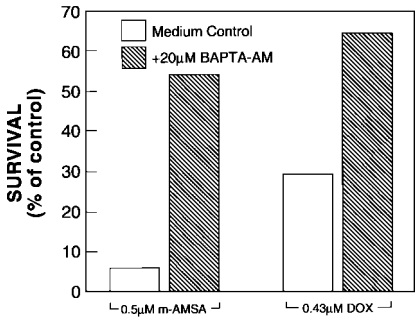

Figure 5 Survival in a soft-agar colony assay of HL-60 cells pretreated with 10 μM BAPTA-AM for 2 h followed by m-AMSA or doxorubicin (DOX) for an additional 1 h

Colony-forming efficiency in the control cells or cells treated with BAPTA-AM alone was 25–30%. Bars indicate S.E.M.

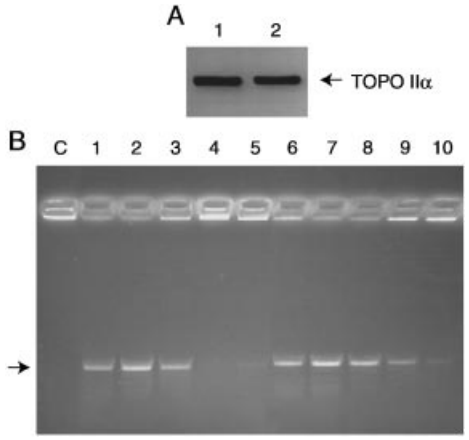

Figure 6 (A) Topo IIα protein in cell lysates and (B) decatenating activity in nuclear extracts from control and BAPTA-AM-treated cells

(**A**) Immunoblot analysis of topo IIα in HL-60 cells treated for 2 h with 20 μM BAPTA-AM. Lysates (150 μg of protein) from control (lane 1) and BAPTA-AM-treated (lane 2) HL-60 cells were resolved by SDS/PAGE, electroblotted on to nitrocellulose and probed with an antiserum specific for topo IIα (at 1:1000 dilution). The immunoreactive protein was detected with an ECL kit (Amersham Life Science Inc.). The integrated density values obtained by densitometric scanning of lanes 1 and 2 were 21252 units and 19964 units respectively. (**B**) Analysis of decatenating activity in nuclear extracts from control (lanes 1–5) and BAPTA-AM-treated (lanes 6–10) HL-60 cells. The substrate, 200 ng of kDNA (Topogen Inc.), was incubated for 30 min with nuclear extracts that were serially diluted to give the following dilutions: lanes 1 and 6, 1:8; lanes 2 and 7, 1:16; lanes 3 and 8, 1:32; lanes 4 and 9, 1:64; lanes 5 and 10, 1:128. The substrate kDNA incubated under similar conditions in the absence of nuclear extract is present in lane C. The position of the decatenated kDNA is indicated by the arrow.

following pretreatment with either 20 μM BAPTA-AM for 1 h or 10 μM BAPTA-AM for 2 h.

Since pretreatment of HL-60 cells with BAPTA-AM significantly inhibited drug-induced DNA damage and cytotoxicity, we sought to examine whether this effect was due to a decrease in topo IIα protein levels and/or altered activity of topo II in the nucleus. The amount of topo IIα protein in cell lysates and the decatenating activity in nuclear extracts from control and BAPTA-AM-treated cells are shown in Figure 6. The data demonstrate that pretreatment of HL-60 cells with BAPTA-AM did not affect the total levels of topo IIα protein (Figure 6A). Further, the catalytic activity of the topo II enzyme, based on the decatenation of kDNA, was comparable in control and BAPTA-AM-treated cells (Figure 6B).

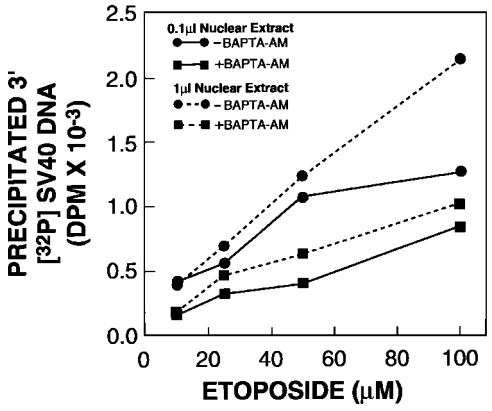

Figure 7 Effect of VP-16 (etoposide) on precipitation of 3′-end-^{32}P-labelled SV40 DNA by nuclear extracts from control and BAPTA-AM-treated HL-60 cells

The nuclear extracts and 3′-end-^{32}P-labelled SV40 DNA in the absence of BAPTA were incubated with VP-16 for 30 min at 37 °C. The reaction was stopped following addition of SDS and the ^{32}P-labelled SV40 DNA–protein complex was precipitated, washed and processed as outlined in the Materials and methods section. The data are expressed as d.p.m. of ^{32}P-labelled SV40 DNA precipitated in the presence of drug minus that precipitated in the absence of drug.

Although cytosolic factors have been reported to enhance drug-stimulated topo II-mediated DNA damage [17–19], nuclear extracts evaluated *ex vivo* in a cell-free system for drug-stimulated protein–DNA complex formation are informative for the characterization of drug-resistant topo II [12,13]. The precipitation of 3′-end-^{32}P-labelled SV40 DNA stabilized by VP-16 in a cell-free system using nuclear extracts from control and BAPTA-AM-treated HL-60 cells is shown in Figure 7. The data demonstrate that, similar to the effects of BAPTA-AM on attenuating the stimulation by VP-16 of topo II-mediated DNA scission in cells, nuclear extracts from BAPTA-AM-treated cells were markedly less effective in stimulating protein–DNA complex formation than those from control untreated cells (Figure 7).

Since treatment of HL-60 cells with BAPTA-AM can significantly reduce the DNA-damaging and cytotoxic potential of topo II poisons (compared with the effect of TPEN), and since nuclear extracts of BAPTA-AM-treated HL-60 cells are markedly less effective in the stabilization of drug-stimulated protein–DNA complexes *ex vivo*, we investigated whether these agents affected the phosphorylation state of topo IIα in cells metabolically labelled with [^{32}P]P_i. The results in Figure 8 from a representative experiment demonstrate that the phosphorylation of topo IIα in control cells was only 1.1-fold greater than that in TPEN-treated cells. However, the phosphorylation of topo IIα in control cells was 1.5-fold greater than that in BAPTA-AM-treated cells. These results thus demonstrate that treatment with BAPTA-AM, but not TPEN, can induce the hypophosphorylation of the topo IIα protein.

The site-specific hypophosphorylation of topo IIα in doxorubicin-resistant HL-60 cells has been reported previously [8]. Since BAPTA-AM treatment of wild-type HL-60 cells both mimics the phenotype of decreased VP-16-induced DNA damage and cytotoxicity characteristic of doxorubicin-resistant HL-60 cells and induces the hypophosphorylation of topo IIα in HL-60 cells, we sought to compare, by using two-dimensional tryptic phosphopeptide mapping, immunoprecipitates of topo IIα from control or BAPTA-AM-treated HL-60 cells with those from

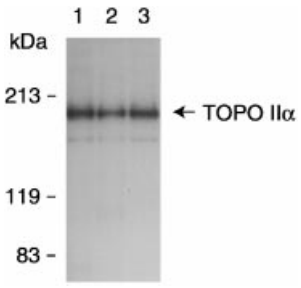

Figure 8 Phosphorylated topo IIα in BAPTA-AM- or TPEN-treated HL-60 cells

Cells either untreated (lane 1) or treated with 20 μM BAPTA-AM (lane 2) or 20 μM TPEN (lane 3) for 1 h were metabolically labelled with [^{32}P]P_i. Nuclei were isolated from 4×10^6 cells, and lysates in RIPA buffer were 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 1.5-fold (range 1.4–1.5) and 1.1-fold (range 1.08–1.13) higher than in BAPTA-AM- and TPEN-treated cells respectively.

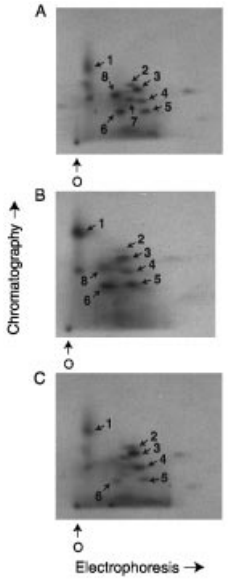

Figure 9 Representative two-dimensional tryptic phosphopeptide maps of topo IIα protein from HL-60 cells (A), HL-60 cells treated with 20 μM BAPTA-AM (B) and topo II-poison-resistant HL-60 cells (C) metabolically labelled with [^{32}P]P_i

Nuclei were isolated from 4×10^6 cells, and lysates in RIPA buffer were immuoprecipitated with antiserum specific for topo IIα. Following digestion with trypsin, washing and lyophilization, phosphopeptides containing 341 d.p.m. (based on Čerenkov counting) were separated horizontally by electrophoresis at pH 1.9 and vertically by chromatography, as described in the Materials and methods section. The phosphorylation sites in HL-60 cells are numbered 1–8; sites 1 and 3 are serine-1524 and serine-1376 respectively, previously identified to be substrates for casein kinase II [16]. O, origin.

doxorubicin-resistant HL-60 cells. The data in Figure 9 demonstrate that in the untreated (control) HL-60 cells there were eight distinct and readily detectable phosphorylation sites (numbered 1–8). In HL-60 cells treated with BAPTA-AM, sites 7 and 8 were the predominant sites that were hypophosphorylated compared with the other detectable phosphorylation sites in untreated (control) HL-60 cells. Since site-specific hypophosphorylation of topo IIα is observed in doxorubicin-resistant HL-60 cells [8], it is remarkable that the four hypophosphorylated sites also include sites 7 and 8. Overall, similar site-specific hypophosphorylation of topo IIα is apparent in BAPTA-AM-treated wild-type HL-60 cells and in doxorubicin-resistant HL-60 cells that are resistant to a variety of topo II posions.

DISCUSSION

Few mechanistic studies have critically addressed the regulation of topo IIα in drug-induced topo II–DNA cleavable complex formation and its correlation with a cytotoxic response in whole-cell systems. Potential mechanisms affecting the reversibility of drug-induced topo II-mediated topo II–DNA cleavable complex formation [20] have been reported, and in resistant sublines a possible role for cleavable complex instability has been suggested [21]. The present data demonstrate that buffering of the intracellular free Ca^{2+} in wild-type human leukaemia HL-60 cells with the Ca^{2+} chelator BAPTA-AM [9] results in: (a) the site-specific hypophosphorylation of the topo IIα protein; and (b) a decrease in drug-stimulated topo II-mediated DNA scission, accompanied by a striking decrease in cytotoxicity. The hypophosphorylation of topo IIα following treatment of HL-60 cells with BAPTA-AM is remarkable, since the two hypophosphorylated sites in the topo IIα protein following treatment of HL-60 cells with BAPTA-AM are similar to two of the four hypophosphorylated sites observed in doxorubicin-resistant cells.

The BAPTA buffers are highly selective for Ca^{2+} over Mg^{2+}, and thus can be used to control both extracellular and intracellular Ca^{2+} [9]. At the resting intracellular Ca^{2+} level, a high selectivity ($> 10^5$) for Ca^{2+} over Mg^{2+} is exhibited by BAPTA. The AM esters of BAPTA also facilitate cell loading. BAPTA has been used to clamp the intracellular Ca^{2+} concentration, thus providing insight into the role of free cytosolic Ca^{2+} [9,10]. In the present study, TPEN has been used as a control for BAPTA, since it binds heavy metals without disturbing Ca^{2+} or Mg^{2+}, and is a useful control for artifacts that may arise from heavy-metal binding by the BAPTA buffers [10]. Comparative studies with BAPTA and TPEN have proven useful in demonstrating that the effects of BAPTA on mitotic progression in Swiss 3T3 fibroblasts are due to specific effects of Ca^{2+} buffering [10]. The comparative effects of BAPTA and TPEN in the present study suggest a functional role for Ca^{2+} in stimulating topo II-mediated DNA scission induced by VP-16 or m-AMSA. The selective effects of BAPTA on attenuating the DNA damage induced by, and cytotoxic potential of, topo II poisons is not due to altered drug accumulation, since, following treatment with 5, 10 and 40 μM VP-16 for 1 h, cellular levels were 21.8, 28.5 and 125.3 nmol of VP-16/10^6 cells respectively in untreated cells, and 22.6, 35.2 and 102.9 nmol/10^6 cells respectively in BAPTA-AM-pretreated cells. The reduced DNA damage following BAPTA-AM treatment is also not due to a decrease in topo IIα protein levels, as shown in Figure 6(A). Treatment of HL-60 cells with BAPTA-AM also does not appear to cause cellular compartmentalization of topo IIα, since comparable data on topo II protein levels were also obtained with isolated nuclei (results not shown). The effects of BAPTA-AM in altering topo II function in nuclear extracts appear to be selective in affecting DNA cleavage (Figure 7) rather than decatenating activity measured with kDNA as the substrate (Figure 6B).

The results from the present study support the hypothesis that hypophosphorylation of topo IIα can significantly decrease drug-stabilized topo II–DNA cleavable complex formation. The role of intracellular Ca^{2+} in inducing the hypophosphorylation of topo IIα is substantiated by the effects of treatment with the Ca^{2+} buffer BAPTA-AM compared with those of TPEN (Figure 8). It might be argued that the decrease in DNA cleavage caused by

Table 1 Characteristics of topo IIα phosphorylation and VP-16-stabilized topo II–DNA cleavable complex formation in wild-type HL-60 cells treated with BAPTA-AM and in doxorubicin-resistant HL-60 cells

Data for HL-60/DOX0.05 cells are from [8].

Cell line	Phosphorylation of topo IIα (170 kDa) (%)	Phosphorylated topo IIα peptides	Increase in topo II–DNA cleavable complex induced by 10 μM VP-16 (fold)
HL-60 (sensitive)	100	Peptides 1–8 phosphorylated	10.9
HL-60 (sensitive) + 20 μM BAPTA-AM	33	Peptides 7 and 8 hypophosphorylated	2.3
HL-60/DOX0.05 (resistant)	45	Peptides 5–8 hypophosphorylated	2.4

BAPTA-AM is due to its pleiotropic cellular effects, and not primarily to alterations in the phosphorylation state of topo II. However, the data in Figure 7 using an *ex vivo* cell-free model system clearly demonstrate a marked decrease in the precipitation of VP-16-induced ^{32}P-labelled SV40-DNA–protein complexes by nuclear extracts from BAPTA-AM-treated compared with untreated (control) cells. Since the topo II in nuclear extracts from BAPTA-AM-treated cells was hypophosphorylated and exhibited decreased drug DNA–protein complex formation over the 10-fold range of protein concentrations tested, this suggests an essential role for phosphorylation in topo II–DNA cleavable complex formation. Nuclear extracts also contain topo IIβ, and we have observed in ongoing studies (results not shown) comparable levels of topo IIα and topo IIβ hypophosphorylation in BAPTA-AM-treated HL-60 cells. A functional role for the phosphorylation state of topo II in drug-stimulated DNA cleavage (Figure 7), but not in the decatenation of kDNA (Figure 6B), is not entirely surprising, since recent studies have demonstrated phosphorylation-independent decatenating activity of topo II [14,22].

The phosphorylation of topo IIα and its role in drug-stabilized topo II–DNA cleavable complex formation, based on comparative studies with BAPTA-AM-treated wild-type HL-60 cells and doxorubicin-resistant HL-60 cells, is summarized in Table 1. Although BAPTA-AM induces hypophosphorylation of topo IIα, the tryptic phosphopeptide maps demonstrate the predominant hypophosphorylation of only two (sites 7 and 8) of the eight phosphorylation sites. The involvement of these hypophosphorylated sites in altering the DNA damage and cytotoxicity induced by VP-16 or m-AMSA in BAPTA-AM-treated wild-type HL-60 cells is supported by the characteristic hypophosphorylation of sites 7 and 8 in HL-60 cells that are resistant to topo II poisons [8]. The hypophosphorylated sites in the doxorubicin-resistant subline [8] or in sensitive HL-60 cells treated with BAPTA-AM are not the residues serine-1376 (site 3) and serine-1524 (site 1) reported previously [16] to be substrates *in vitro* and *in vivo* for casein kinase II. Because of potential problems that can arise with the extractability of topo II and the recovery of peptides during extraction and preparation, tryptic phosphopeptide mapping was carried out by loading similar d.p.m. (based on Čerenkov counting) for each sample. It appears unlikely that technical problems may contribute to the observation of site-specific hypophosphorylation in Figure 9, since the results demonstrate that, in spite of the different treatments and cell types evaluated, the signal intensity is comparable for site 1 (serine-1524) and site 3 (serine-1376), which are substrates for casein kinase II [16].

Sensitivity to anti-tumour drugs has been reported to be a function of the intracellular distribution of topo II during cell-cycle progression and of the topo II associated with the high-salt-insoluble nuclear matrix [23,24]. Since intracellular Ca^{2+} transients, which are essential for cell cycle progression, are inhibited by BAPTA-AM treatment, the cellular distribution and extractability of topo II could be affected. However, in previous studies with doxorubicin-resistant cells [8], as well as in the present study with untreated and BAPTA-AM-treated wild-type HL-60 cells, the extractable protein (determined by Coomassie protein assay), as well as topo IIα and topo IIβ levels (determined by immunoblotting), were similar in lysates of isolated nuclei. Also, results of cell cycle phase distribution analyses (by flow cytometry) of control and BAPTA-AM-treated HL-60 cells were not remarkably different. The cellular effects of BAPTA-AM treatment were also reversible, although some increase in cell generation time was observed. The effects of BAPTA-AM in attenuating drug-stabilized topo II–DNA cleavable complex formation and cytotoxicity were maximal with pretreatment rather than with simultaneous exposure.

Studies with diverse cell model systems that are resistant to topo II poisons have suggested the potential importance of the hypophosphorylated state of topo IIα [6–8,25]. Since tumour cell resistance can be multifactorial, the interpretation of results with resistant cells can be problematic. However, the data in the present study demonstrating that the characteristic site-specific hypophosphorylation of topo IIα and reduced drug-stabilized topo II–DNA cleavable complex formation can be mimicked by treating wild-type cells with BAPTA-AM suggest an essential role for topo II phosphorylation in eliciting drug-induced DNA damage and cytotoxicity. A role for Ca^{2+} and altered topo II phosphorylation with BAPTA-AM treatment is also supported by our recent data demonstrating that intracellular free Ca^{2+} transients are required for the sensitization of tumour cells resistant to topo II poison by inhibitors of Ca^{2+}/calmodulin-dependent enzymes [26].

In summary, we demonstrate that the phosphorylation state of topo IIα is possibly important in drug-stabilized topo II-mediated DNA cleavage. While these results propose a central role for topo IIα, a possible contribution of topo IIβ cannot be discounted, since BAPTA-AM also induces hypophosphorylation of this isoform. Since the correlation between reduced DNA cleavage and cytotoxicity is striking on manipulation of the phosphorylation of topo II, a functional role for site-specific DNA cleavage in the induction of apoptosis and cytotoxicity by topo II poisons can now be addressed. We can also now determine, via functional experiments, whether the phosphorylation of site 7 and/or site 8 plays an essential role in cell regulation or tumour cell resistance to topo II poisons.

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