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Overexpression of the RIα Subunit of Protein Kinase A Confers Hypersensitivity to Topoisomerase II Inhibitors and 8-Chloro-cyclic Adenosine 3′,5′-Monophosphate in Chinese Hamster Ovary Cells[1]

Phillip S. North, Sally L. Davies, Fortunato Ciardiello, Vincenzo Damiano, Caterina Bianco, Stefano Pepe, A. Raffaele Bianco, Adrian L. Harris, Ian D. Hickson, and Giampaolo Tortora[2]

Imperial Cancer Research Fund, Institute of Molecular Medicine, University of Oxford, Oxford OX3 9DU, United Kingdom [P. S. N., S. L. D., A. L. H., I. D. H.], and Cattedra di Oncologia Medica, Facoltà di Medicina, Università di Napoli Federico II, Napoli 80131, Italy [F. C., V. D., C. B., S. P., A. R. B., G. T.]

ABSTRACT

We have shown that a mutant derivative of Chinese hamster ovary CHO-K1 cells, ADR-5, which shows hypersensitivity to topoisomerase II (topo II)-inhibitory drugs, is cross-sensitive to the site-selective cyclic AMP analogue 8-chloro-cyclic AMP. We tested the hypothesis that overexpression of the type Iα regulatory subunit of protein kinase A may represent a common element conferring hypersensitivity to both topo II inhibitors and 8-chloro-cyclic AMP in ADR-5 cells. We have demonstrated that ADR-5 cells overexpress RIα protein, compared to parental CHO-K1 cells. Moreover, retroviral vector-mediated transfer of the RIα gene into CHO-K1 cells was able to confer a drug-hypersensitive phenotype similar to that exhibited by ADR-5 cells. Analysis of topo II protein levels and activity revealed no differences between parental and infected cells, suggesting that protein kinase A may be involved in the downstream processing of topo II-mediated events.

INTRODUCTION

Topo II[3] is a nuclear enzyme which catalyzes the concerted breakage of double-stranded DNA and passage of an intact second duplex through the break (1–3). Topo II is a constituent of the mitotic chromosome scaffold (4) and has an essential role in cell proliferation (5), catalyzing the segregation of replicated DNA at mitosis (6–8). In mammalian cells, there are two closely related isoforms of topo II, designated α and β, which are encoded on different chromosomes (9–11) and are apparently differentially regulated (12–14). Several anticancer drugs, including the nonintercalating epipodophyllotoxins etoposide and teniposide, exert their cytotoxicity at least in part by inhibition of topo II. These drugs stabilize a covalent DNA-protein complex, termed the cleavable complex, in which a topo II monomer is attached via an active site tyrosine to each 5′-phosphoryl end of a double-stranded break in the DNA (15–18). These lesions lead eventually to cell death by an unknown mechanism. It has been shown previously that differential sensitivity and/or resistance of cancer cells to topo II-inhibitory drugs may depend upon changes in the levels of expression and/or the activity of topo II protein (19–23). However, a mutant derivative of CHO-K1 cells, designated ADR-5, has been isolated which shows hypersensitivity to topo II inhibitors but does not appear to have abnormal topo II levels or enzymatic activity (24).

cAMP acts in mammalian cells by binding to either of two distinct isoforms of PKA, defined as PKAI and PKAII (25). The isozymes differ in their cAMP-binding regulatory subunits (termed RI in PKAI and RII in PKAII) but share common catalytic subunits (25). Differential expression of PKAI and PKAII has been correlated with cell differentiation and neoplastic transformation (26). Indeed, preferential expression of PKAII is observed in normal nonproliferating tissues and in growth-arrested cells, while enhanced levels of the RI subunit, namely the RIα isoform, and/or the PKAI holoenzyme are generally found in tumor cells and in normal cells following exposure to mitogenic stimuli (27–31). Moreover, RIα has recently been implicated in the regulation of the transition from G_1 to S phase of the cell cycle and has been identified as the extinguisher of differentiation (29, 32, 33). The site-selective cAMP analogue 8-Cl-cAMP causes inhibition of RIα expression, induction of RIIβ, and cell growth arrest in a large variety of cancer cells *in vitro* and *in vivo* and is currently under investigation in phase I clinical trials in cancer patients (34).

Here we show that the ADR-5 mutant derivative of CHO-K1 cells is also hypersensitive to 8-Cl-cAMP. This led us to speculate whether ADR-5 cells overexpress the RIα subunit of PKA. This was demonstrated to be the case. To confirm that RIα protein overexpression was directly involved in the phenotype of ADR-5 cells, the RIα gene was expressed from a retroviral construct in parental CHO-K1 cells. The infected cells showed hypersensitivity to both topo II poisons and 8-Cl-cAMP but did not appear to have abnormal topo II levels or activity.

MATERIALS AND METHODS

Cell Cultures. CHO-K1, ADR-5, CHO-neo, CHO-RIα, CHO-RIIβ, and CHO-Cα cells were grown in Ham's F10 medium supplemented with 5% heat-inactivated newborn calf serum, 5% fetal bovine serum, 5 mM glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin (ICN Biomedicals, Costa Mesa, CA), in a humified atmosphere of 95% air/5% CO_2 at 37°C. ADR-5 is a clonal cell line generated as described previously (24). CHO-RIα, CHO-RIIβ, CHO-Cα, and CHO-neo cells were generated by infection with recombinant amphotropic retroviral vectors containing the neomycin resistance gene and the full length complementary DNAs encoding the RIα, RIIβ, or Cα genes or the same vector containing the neomycin resistance gene alone (35, 36).

To evaluate the effects of 8-Cl-cAMP on cell growth, exponentially growing cells were trypsinized and seeded in duplicate 35-mm dishes (Becton Dickinson, Milan, Italy). Following a 4-h incubation to permit attachment to the dishes, the medium was replaced with either control medium or medium containing 8-Cl-cAMP. At different times after drug exposure, cells were trypsinized and counted with an hemocytometer. For clonogenic assays cells were seeded in triplicate as described above, to yield 2–3 × 10^2 cells. Following treatment with 8-Cl-cAMP as described above, the dishes were incubated at 37°C for 10–12 days for colonies to develop. These were fixed in methanol:acetic acid (3:1), stained with crystal violet (400 μg/ml), and counted. Colonies containing more than 50 cells were considered survivors.

Measurement of Cytotoxic Drug Sensitivity. Cells (10^3) were plated in 96-well clusters (Becton Dickinson) and treated with different concentrations of etoposide, teniposide, CDDP, or bleomycin (Sigma Chemical Co., St Louis,

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[2] To whom requests for reprints should be addressed, at Cattedra di Oncologia Medica, Facoltà di Medicina, Università di Napoli Federico II, Via S. Pansini, 5, 80131 Napoli, Italy.

[3] The abbreviations used are: topo II, topoisomerase II; PKA, cAMP-dependent protein kinase; CDDP, *cis*-diaminodichloroplatinum; PKAI, cAMP-dependent protein kinase type I; PKAII, cAMP-dependent protein kinase type II; CHO, Chinese hamster ovary; cAMP, cyclic AMP; IC_{50}, 50% inhibitory concentration; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; gp170, M_r 170,000 glycoprotein; 8-Cl-cAMP, 8-chloro-cyclic AMP.

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MO) for 1 h. Stock solutions (100×) of drugs were prepared immediately before use and diluted in medium. Treatments were carried out as described previously (21) and after 6 days of culture the cells were incubated at 37°C with dimethylthiazol diphenyltetrazolium bromide for 4 h and readings of absorbance were carried out as described (21). For clonogenic assays, 500 cells were plated in 35-mm dishes (Becton Dickinson) and treated with the appropriate drug concentrations for 24 h, and colonies were counted after 10–12 days as described above. To determine the IC_{50} values, linear regressions were plotted for the linear region of the curves. Mean values were calculated from a minimum of three experiments, each performed in six replicates for each drug and cell line.

Western Blot Analysis. Protein lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (50 μg total proteins/lane) on 4–20% gradient gels (Bio-Rad Laboratories, Milan, Italy) and transferred to nitrocellulose, and the filters were incubated with a mouse monoclonal anti-human RIα antibody (kindly provided by Dr. B. Skhalegg, Rickshospitalet, Oslo, Norway), as described previously (37).

Determination of Topo II Levels by Flow Cytometry. Cells were trypsinized, washed twice with Ca^{2+}/Mg^{2+}-free PBS, and fixed in 100% methanol for 10 min at −20°C. Cells (10^6) were incubated with 2 ml of neutralizing buffer (Ca^{2+}/Mg^{2+}-free PBS plus 2% goat serum) at room temperature for 15 min. After washing twice with Ca^{2+}/Mg^{2+}-free PBS, 50 μl of anti-topo II rabbit antibody T2K1 (21), diluted 1:10 in Ca^{2+}/Mg^{2+}-free PBS plus 2% bovine serum albumin, were added to each test sample. Negative control samples were incubated with preimmune rabbit serum instead of the anti-topo II antibody. After 60 min at room temperature, the samples were washed twice with Ca^{2+}/Mg^{2+}-free PBS, and 100 μl of FITC-conjugated goat-anti rabbit antiserum (Sigma) (diluted 1:30 in Ca^{2+}/Mg^{2+}-free PBS plus 2% bovine serum albumin) were added to the samples. To determine the gp170 content, cells were harvested and washed as described above, fixed in 70% methanol, and incubated with 50 μl of anti-gp170 FITC-conjugated mouse monoclonal antibody P-glycoCHEK C219, while negative control samples were incubated with 50 μl of FITC-conjugated mouse P-glycoCHEK negative antibody (Centocor Diagnostics Inc., Malvern, PA).

Flow cytometric analysis was performed in duplicate with a FACScan flow cytometer (Becton Dickinson, San Jose, CA) coupled to a Hewlett-Packard computer. A CONSORT 30 data analysis system (Becton Dickinson) was utilized for data acquisition.

Decatenation Assay of Plasmid DNA. Topo II catalytic activity was measured in CHO-K1 and CHO-RIα nuclear cell extracts as the rate of decatenation of kinetoplast DNA from *Crithidia fasciculata*. Agarose gel electrophoresis and DNA visualization were carried out as described previously (19).

RESULTS

ADR-5 cells are hypersensitive to topo II inhibitors but do not appear to have abnormal topo II content or activity. Moreover, no differences have been found in the content of gp170, the product of the multidrug resistance gene, compared to parental CHO-K1 cells (data not shown).

We first compared the response of CHO-K1 and mutant ADR-5 cells to the growth-inhibitory effects of 8-Cl-cAMP. Fig. 1*A* shows that, while 50 μM 8-Cl-cAMP had only a moderate effect on the growth rate of parental CHO-K1 cells, this concentration caused a marked inhibition of ADR-5 cell growth. Moreover, clonogenic assays performed following treatment with 8-Cl-cAMP showed that ADR-5 cells were approximately 10-fold more sensitive to the drug than were CHO-K1 cells, based on IC_{50} values (Fig. 1*B*).

To determine whether this differential sensitivity may be due to increased expression of PKAI, the proposed target for 8-Cl-cAMP, we measured the expression of the RIα subunit of PKA in CHO-K1 and ADR-5 cells by Western blotting. As shown in Fig. 2, ADR-5 cells exhibited an 8-fold higher RIα protein level than did parental CHO-K1 cells.

It has been demonstrated previously that ADR-5 cells are hypersensitive to topo II-targeted drugs (24). To verify that the increase in RIα protein levels in ADR-5 cells was directly associated with hypersensitivity to topo II-targeted drugs, we infected parental CHO-K1 cells with a recombinant retroviral vector to overexpress the RIα subunit of PKA. As controls, vectors expressing the RIIβ or Cα proteins were introduced into CHO-K1 cells. The CHO-RIα, CHO-RIIβ, and CHO-Cα infectants, which were screened by Northern blot analysis for expression of the respective transduced genes following stimulation with 1 μM $CdCl_2$ (data not shown), were also analyzed by Western blotting to determine the levels of RIα protein expression (Fig. 2). CHO-RIα cells treated with $CdCl_2$ (1 μM for 48 h) exhibited a 15-fold increased level of RIα protein, compared to either CHO-K1 cells (Fig. 2) or CHO-neo cells (data not shown). In contrast, CHO-Cα cells expressed RIα protein levels comparable to those of parental CHO-K1 cells, while CHO-RIIβ cells showed a 2-fold reduction (Fig. 2). In addition, RIα protein levels were not modified in CHO-K1 and CHO-neo cells following $CdCl_2$ treatment (data not shown). Of

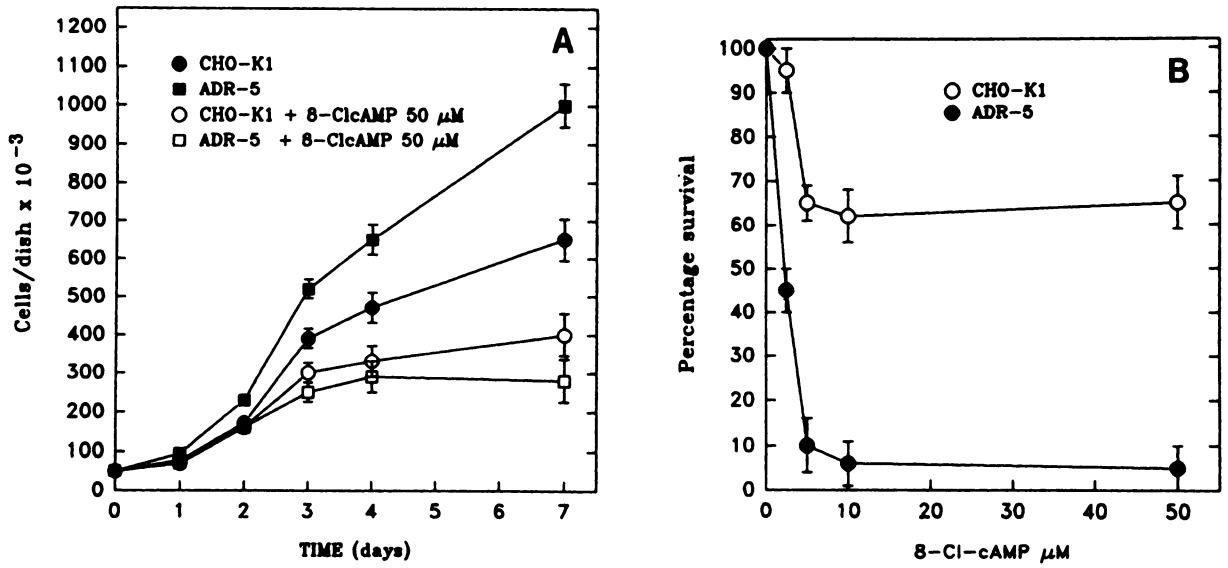

Fig. 1. Effect of 8-Cl-cAMP on CHO-K1 and ADR-5 cell growth and clonogenic survival. *A*, growth curves for CHO-K1 and ADR-5 cells in the presence and absence of 50 μM 8-Cl-cAMP. *B*, clonogenic survival of CHO-K1 and ADR-5 cells following exposure to 50 μM 8-Cl-cAMP. All cells were grown in the presence of $CdCl_2$.

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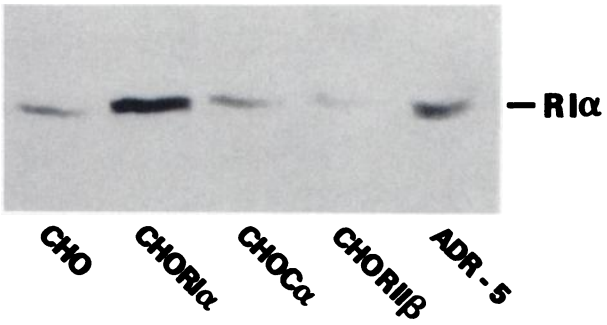

Fig. 2. Western blotting analysis of RIα expression in cell extracts, using a monoclonal antibody directed against the M_r 49,000 RIα subunit of PKAI. Blots were performed as described in "Materials and Methods."

the infected cells, only CHO-RIα cells exhibited normal growth properties, while the CHO-RIIβ and CHO-Cα cells had a longer doubling time.[4]

We next studied the effects of 8-Cl-cAMP treatment on CHO-K1 and CHO-RIα cells that were grown in the presence of 1 μM $CdCl_2$. While 8-Cl-cAMP at 50 μM produced only approximately 30% growth inhibition in CHO-K1 cells, >75% growth inhibition was observed after 7 days of drug treatment in CHO-RIα cells (Fig. 3*A*). Moreover, a clonogenic assay demonstrated that >90% of CHO-RIα cells were inhibited by 8-Cl-cAMP treatment (Fig. 3*B*).

Moreover, in the absence of $CdCl_2$, CHO-RIα cells showed a growth pattern similar to that of parental CHO-K1 cells (data not shown). To test whether CHO-RIα cells were hypersensitive to topo II-inhibitory drugs, we treated CHO-K1 and CHO-RIα cells with various cytotoxic drugs and performed clonogenic assays. A 2.5-fold lower IC_{50} value for etoposide was seen in the CHO-RIα cells (2 μg/ml), compared to CHO-K1 cells (5 μg/ml) (Fig. 4). CHO-RIα cells also showed a 2-fold higher level of sensitivity to teniposide, with an IC_{50} of 0.6 μg/ml (data not shown). In contrast, CHO-K1 and CHO-RIα cells showed similar levels of sensitivity to CDDP, with IC_{50} values of 4.2 and 4.0 μg/ml, respectively (Fig. 4), and to bleomycin (IC_{50} of 75 μg/ml for each cell line; data not shown). Thus, CHO-RIα cells showed hypersensitivity to epipodophyllotoxins which target topo II but not to CDDP or bleomycin, two drugs which act independently of topo II (15–17, 24). We also examined the cytotoxic effects of etoposide and teniposide treatment on CHO-RIα cells that were grown with or without $CdCl_2$, using a clonogenic assay (Fig. 5). In $CdCl_2$-treated CHO-RIα cells, etoposide and teniposide showed IC_{50} values 3-fold and 2-fold lower, respectively, than those obtained in untreated cells, strongly suggesting that the induction of RIα gene expression by $CdCl_2$ was responsible for the increased sensitivity of these cells to the topo II-targeted drugs (Fig. 5).

To evaluate whether the hypersensitivity of CHO-RIα cells to topo II inhibitors could be related to an increase in topo II protein levels, we determined the cellular level of topo IIα protein by flow cytometry, using a specific antibody directed against human topo IIα isozyme (21). As shown in Fig. 6, no differences in topo IIα protein expression were observed in CHO-K1, ADR-5, or CHO-RIα cells.

It has been shown previously that ADR-5 cells possess a topo II activity similar to that of CHO-K1 cells (24). To determine whether any difference in topo II catalytic activity was evident in the CHO-RIα cells, a decatenation assay using catenated kinetoplast DNA and crude nuclear protein extracts was performed. Fig. 7 shows that the time course of decatenation catalyzed by CHO-RIα nuclear extracts was not altered following treatment of the cells with $CdCl_2$, although this treatment induced hypersensitivity to etoposide (see above).

DISCUSSION

Overexpression of the RIα subunit of PKAI has been correlated with enhanced cell proliferation and neoplastic transformation, and it has been proposed that this protein could be a novel target for cancer therapy (26, 27, 34, 37). In this study, we have tested the hypothesis that overexpression of the RIα protein represents a common element conferring upon CHO-K1 cells increased sensitivity to both topo II inhibitors and the site-selective cAMP analogue 8-Cl-cAMP, a potent down-regulator of RIα expression. We have shown that a mutant of CHO-K1 cells that is hypersensitive to topo II-targeted drugs overexpresses the RIα protein, compared to the parental cells. We have also shown that ADR-5 is hypersensitive to the growth-inhibitory effect of 8-Cl-cAMP. Moreover, we have been able to confer upon the parental CHO-K1 cells a drug-hypersensitive phenotype very similar to that of ADR-5 cells by retroviral vector-mediated transfer of the RIα gene. Taken together, these data show that there is a relationship between the level of expression of the RIα protein and cellular sensitivity to both 8-Cl-cAMP and topo II inhibitors. Although there was not a direct quantitative correlation between the amount of RIα and the degree of drug sensitivity, infection of CHO-K1 cells with the RIα expression retroviral vector generated cells with a phenotype very similar to that of ADR-5 cells, showing hypersensitivity to both topo II inhibitors and 8-Cl-cAMP but not to drugs such as CDDP or bleomycin, which do not act via topo II.

It has been shown in several cell lines that levels of topo II may account for differential sensitivity to topoisomerase-inhibitory drugs (19–23). This is apparently not the case for ADR-5 cells, which exhibit a level of topo II activity which is comparable to that seen in parental CHO-K1 cells (24). While our results indicate that there is a correlation between the overexpression of the RIα protein of PKAI and cellular sensitivity to topo II inhibitors, this relationship apparently cannot be explained either by differential expression of topo II protein or by modulation of the activity of topo II in cells overexpressing the RIα protein. This leaves open the question of the mechanism by which RIα protein influences the cellular sensitivity to topo II inhibitors.

It is possible that RIα protein acts directly or indirectly upon topo II-mediated events. For example, it has been shown that PKA (as well as casein kinase II and protein kinase C) is able to phosphorylate topo II on serine/threonine residues and that this modification leads to a stimulation in activity, as measured by the decatenation assay (38–42). Although the effects of PKA phosphorylation on the susceptibility of mammalian cell topo II to inhibition by epipodophyllotoxins have not been determined, the finding that the catalytic activity of topo II is unaltered in cells overexpressing RIα protein suggests that modulation of topo II activity by PKA is unlikely to underlie the effects reported here. However, it is possible that our results reflect changes in the phosphorylation status of topo II which influence susceptibility to drug inhibition but do not alter catalytic activity. An additional explanation of our findings could be related to the role of RIα in the G_1 to S transition of the cell cycle (29). The overexpression of RIα may produce changes in cell cycle distribution and this perturbation may affect sensitivity to topoisomerase II-directed drugs.

It has also been reported that topo II may interact directly with a cAMP-responsive element-binding protein and other related transcription factors that could affect the transcription of cAMP-dependent genes (43). The overexpression of RIα protein may alter this putative interaction between cAMP-responsive element-binding protein and

[4] G. Tortora, S. Pepe, C. Bianco, V. Damiano, A. Ruggiero, G. Baldassarre, C. Corbo, Y. S. Cho-Chung, A. R. Bianco, and F. Ciardiello. Differential effects of protein kinase A subunits on Chinese hamster ovary cell cycle and proliferation, submitted for publication.

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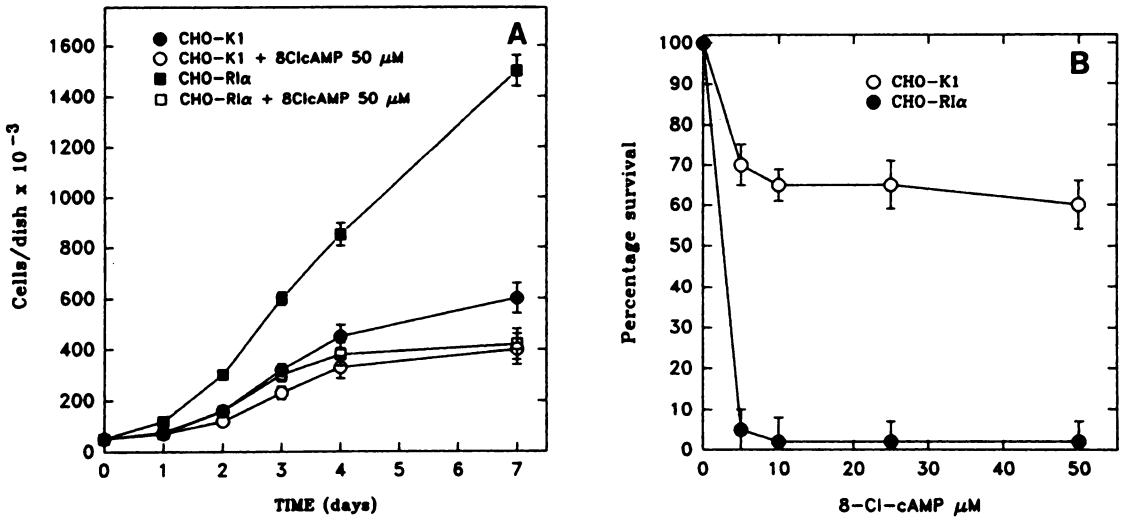

Fig. 3. Effect of 8-Cl-cAMP treatment on CHO-K1 and CHO-RIα cell growth. *A*, growth curves for cells grown in the presence or absence of 50 μM 8-Cl-cAMP for 7 days. Cell numbers were determined as described in "Materials and Methods." *B*, clonogenic survival of CHO-K1 and ADR-5 cells following exposure to 50 μM 8-Cl-cAMP. All cells were grown in the presence of $CdCl_2$.

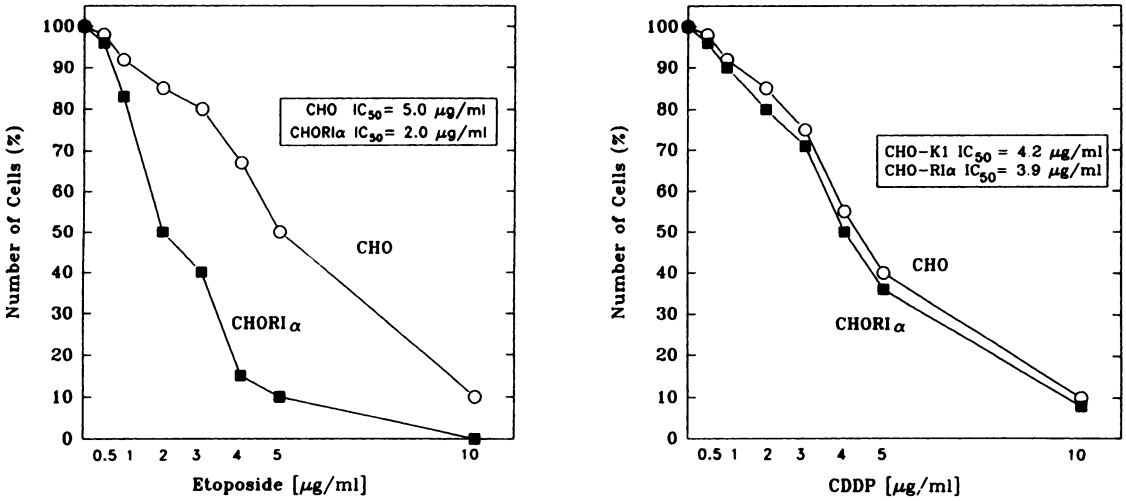

Fig. 4. Differential effect of etoposide and CDDP on CHO-K1 and CHO-RIα cells. Cells were exposed to different concentrations of etoposide (*left*) or CDDP (*right*) and cell numbers were determined using the dimethylthiazol diphenyltetrazolium bromide assay, as described in "Materials and Methods." Data represent one of three different experiments giving similar results. All cells were grown in the presence of $CdCl_2$.

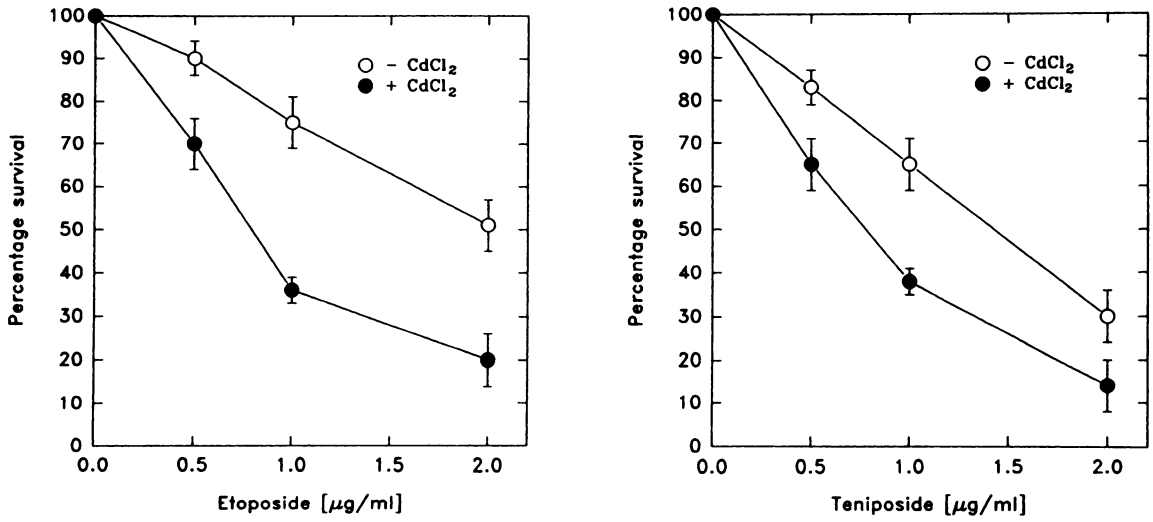

Fig. 5. Effect of etoposide and teniposide on CHO-RIα colony formation, with or without $CdCl_2$. Clonogenic survival of CHO-RIα cells was measured following exposure to etoposide (*left*) or teniposide (*right*). Cells were pretreated (●) or not (○) with 1 μM $CdCl_2$ prior to the survival analyses were performed. Data represent the average ± SD of two different experiments.

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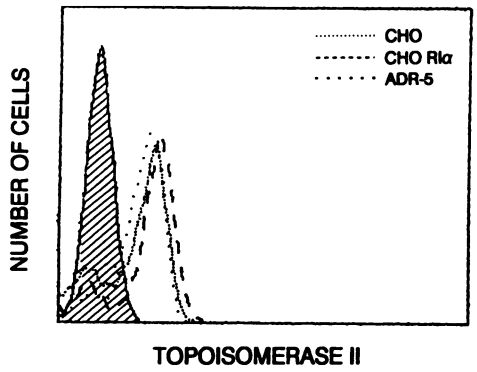

Fig. 6. Flow cytometric analysis of topoisomerase II levels. The content of topo II, compared to DNA content, was measured in CHO-K1, ADR-5, and CHO-RIα cells as described in "Materials and Methods." *Shaded area* represents nonspecific fluorescence.

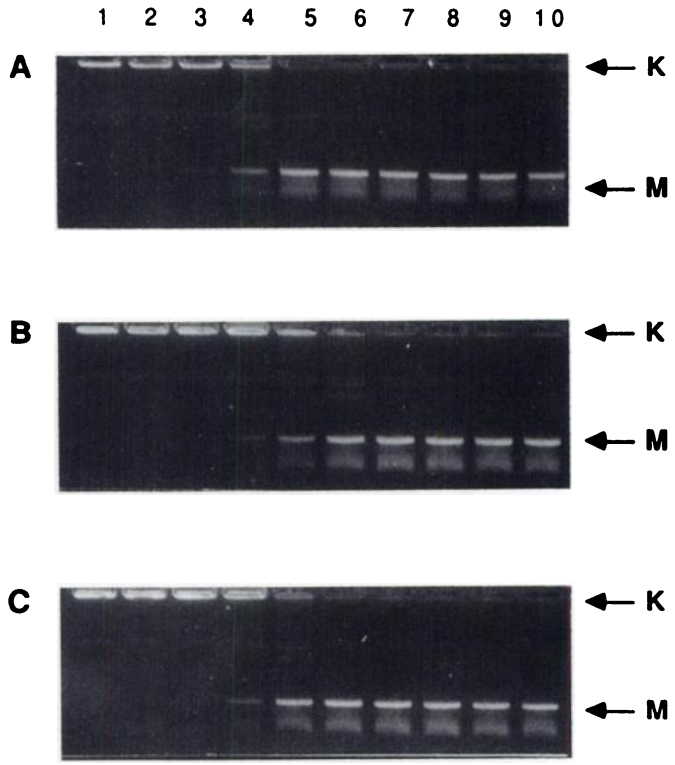

Fig. 7. Analysis of topo II activity by decatenation assay. Decatenation of kinetoplast DNA (*K*) to mini-circles (*M*) by nuclear extracts from CHO-neo cells (*A*), CHO-RIα cells grown in the absence of $CdCl_2$ (*B*), and CHO-RIα cells grown in the presence of $CdCl_2$ (*C*) was measured. *Lane 1*, time 0; *lane 2*, 2.5 min; *lane 3*, 5 min; *lane 4*, 7.5 min; *lane 5*, 10 min; *lane 6*, 12.5 min; *lane 7*, 15 min; *lane 8*, 20 min; *lane 9*, 25 min; *lane 10*, 30 min.

topo II, which in turn influences gene expression. For this mechanism to be relevant, it is necessary to speculate that one or more genes regulated in this way somehow alter the processing of the topo II-dependent cleavable complex or some other cytotoxic lesions generated by etoposide. Further work is necessary to assess whether this putative mechanism is relevant to the phenotype of ADR-5 cells.

In conclusion, our study suggests that the PKAI isoform, or specifically the RIα subunit of PKAI, may interfere with the processing of topo II-related events and that this may be responsible for the drug-hypersensitive phenotype described in ADR-5 and CHO-RIα cells. Our study may also provide a rationale for the study of combined therapeutic modalities with conventional cytotoxic drugs and modulators of PKA activity, such as 8-Cl-cAMP, which is currently under investigation in phase I clinical trials in patients with cancer.

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Overexpression of the RIα Subunit of Protein Kinase A Confers Hypersensitivity to Topoisomerase II Inhibitors and 8-Chloro-cyclic Adenosine 3′,5′-Monophosphate in Chinese Hamster Ovary Cells

Phillip S. North, Sally L. Davies, Fortunato Ciardiello, et al.

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