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Inhibition of Protein Kinase Cα Enhances Anticancer Agent-Induced Loss of Anchorage-Independent Growth Regardless of Protection against Apoptosis by Bcl-2

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ABSTRACT

In the present study, we investigated the effects of several selective protein kinase C (PKC) inhibitors (Gö6976, Gö6983, bisindolylmaleimide I, and rottlerin) in combination with conventional anticancer drugs on apoptosis and long-term anchorage-independent growth of both parental and Bcl-2-overexpressing mammary adenocarcinoma MTLn3 cells. In normal MTLn3 cells, doxorubicin- and etoposide-induced apoptosis was not affected by any of the PKC inhibitors. However, Bcl-2-mediated cytoprotection against apoptosis was slightly counteracted by Gö6976, a selective inhibitor of PKCα, as well as by transient overexpression of dominant-negative PKCα. Doxorubicin and etoposide both inhibited anchorage-independent growth; for doxorubicin, this occurred at concentrations that did not yet cause apoptosis. Overexpression of Bcl-2 did not overcome these growth-inhibitory effects. The effects of doxorubicin on colony formation were potentiated by Gö6976, Gö6983, and bisindolylmaleimide I but not rottlerin. In contrast, etoposide-induced loss of clonogenicity was primarily enhanced by Gö6976. Gö6976 alone, but not Gö6983, bisindolylmaleimide I, or rottlerin, inhibited colony formation in soft agar. This effect of Gö6976 correlated with inhibition of cell cycle progression. Overall, the data indicate that pharmacological inhibitors of PKCα in combination with anticancer drugs, act additively to inhibit long-term anchorage-independent tumor cell growth, independent of apoptosis induction. Importantly, similar additive effects are observed in Bcl-2 overexpressing cells.

Protein kinase C (PKC) is an important family of signaling molecules that regulate diverse processes in the cell, including proliferation, differentiation, transformation, and apoptosis (Nishizuka, 1995; Kiley et al., 1996; Mellor and Parker, 1998). The effects are determined by differential expression and regulation of the twelve isoenzymes that constitute the PKC family. These are divided into three subclasses, i.e., "classic" (α, $\beta_{I/II}$, and γ), "novel" (δ, ϵ, η, and θ) and "atypical" (ζ and λ/ι) isoenzymes, depending on their requirement of Ca^{2+}, phosphatidylserine, and diacylglycerol (Nishizuka, 1995; Newton, 1997).

PKCs are known for their role in tumorigenesis (Taketani and Oka, 1983; Nishizuka, 1995; Kazanietz, 2000), and changes in PKC activity and PKC isoenzyme expression patterns of, for example, PKCα and PKCδ occur in a variety of tumors, including breast cancer. This is associated with increased tumor growth and metastasis (Ways et al., 1995; Gordge et al., 1996; Kiley et al., 1999a,b). The involvement of PKC isozymes in tumor development/progression has been the rationale for the use of PKC inhibitors and antisense oligonucleotides in tumor treatment. Thus, UCN-01, which inhibits classic PKCs but also the cell cycle regulators CDK-1 and -2 (Akinaga et al., 1994; Wang et al., 1995; Akiyama et al., 1997), or PKCα antisense oligonucleotides induced growth arrest and decreased tumor growth of human tumors in nude mice (Dean et al., 1996; Pollack et al., 1996; Yazaki et al., 1996). However, in clinical trials these compounds only marginally inhibited tumor growth (Flaherty et al., 2001). Therefore, treatment with a combination of both PKC inhibitors and classic anticancer agents may be more promising to defeat cancer. Such a combined treatment may reduce the threshold for the onset of apoptosis or for inhibition of cell proliferation.

The combined treatment with anticancer agents and PKC modulators has been studied in some tumor cell lines, predominantly at the level of apoptosis. In general, it appears that PKCα protects and PKCδ potentiates apoptosis in combination with anticancer drugs. Thus, down-regulation of PKCα has been shown to occur during etoposide-induced

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ABBREVIATIONS: PKC, protein kinase C; Ara-C, 1-β-D-arabinufuranosylcytosine; α-MEM, α-modified minimal essential medium; Bis I, bisindolylmaleimide I; Neo, neomycin-resistant cells; PI, propidium iodide; AMC, 7-amino-4-methylcoumarin; Ac-DEVD-AMC, *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin; GFP, green fluorescent protein; GFP-H2B, GFP-histone 2B; WT, wild type.

apoptosis, and overexpression of wild-type PKCα protects against this effect in Cos cells (Whelan and Parker, 1998). Overexpression of PKCα decreases and overexpression of PKCϵ and ζ increases the sensitivity to cisplatin-induced apoptosis as well as doxorubicin-induced growth inhibition in NIH3T3 cells (Spitaler et al., 1999). PKCδ activity is also increased during etoposide-induced apoptosis; inhibition of PKCδ by either rottlerin or kinase-dead PKCδ protects against etoposide-induced loss of mitochondrial membrane potential and caspase activation in salivary epithelial cells (Reyland et al., 1999; Matassa et al., 2001). Similarly, PKCδ overexpression sensitizes these and glioma cells to etoposide (Matassa et al., 2001; Blass et al., 2002). However, anticancer agents and PKC inhibitors may affect not only apoptosis but also cell turnover/proliferation. However, the above-mentioned studies that assessed the involvement of PKC in anticancer drug-induced apoptosis did not include the long-term effects of combined treatment. For the clinical outcome, it is essential to know to what extent tumor cells are still capable of long-term survival following tumor treatment. Moreover, clinically relevant concentrations of anticancer agents may not always induce apoptosis whereas long-term survival may be affected.

The investigations on the relationship between apoptosis and long-term cell survival/proliferation are to some extent conflicting. For example, Bcl-2 inhibits apoptosis induced by a variety of stimuli, and its overexpression can also induce transformation of cells resulting in increased clonogenicity and tumor growth (Basolo et al., 1999; Miyake et al., 1999). However, Bcl-2 overexpression inhibited apoptosis but did not prevent loss of anchorage-independent growth caused by 1-β-D-arabinofuranosylcytosine (Ara-C) or paclitaxel (Wang et al., 1999; Tang et al., 2000). The fact that Bcl-2 is not always able to restore clonogenicity indicates that the short-term effects on apoptosis do not necessarily result in corresponding effects on long-term survival and metastatic capability of remaining tumor cells. These two studies were performed in leukemic cells. Little is known about the relationship between apoptosis and long-term survival, in general and particularly in relation to Bcl-2-mediated protection against apoptosis, after treatment of other tumor cell types with anticancer agents alone or in combination with PKC inhibitors.

In the present study, we investigated the effect of several PKC inhibitors on both loss of clonogenic potential in soft agar and induction of apoptosis caused by two anticancer agents, doxorubicin and etoposide, which are commonly used in the treatment of breast cancer (Hortobagyi, 1997; Hande, 1998). We used the rat mammary adenocarcinoma cell line MTLn3, which is often used as a model to study molecular mechanisms of metastasis formation (Kiley et al., 1999b) and responses to drug therapy both in vitro and in vivo (Toyota et al., 1998; Huigsloot et al., 2001, 2002) To inhibit apoptosis we also used MTLn3 cells that overexpress Bcl-2. The soft agar colony formation assay estimates the ability of single tumor cells to survive and proliferate in an anchorage-independent manner (both required for tumor growth and metastasis). Therefore, we used this assay to determine the long-term effects of anticancer drugs. Our data indicate that inhibition of PKCα in combination with doxorubicin or etoposide decreased clonogenicity additively. This effect appears to be unrelated to enhancement of apoptosis. In addition, although Bcl-2 prevented the induction of apoptosis by both doxorubicin and etoposide, no restoration of long-term survival of breast cancer cells was observed.

Materials and Methods

Chemicals. α-Modified minimal essential medium (α-MEM) with ribonucleosides and deoxyribonucleosides, fetal bovine serum, penicillin/streptomycin, LipofectAMINE Plus, and geneticin (G418 sulfate) were obtained from Invitrogen (Breda, The Netherlands). Gö6976 and Gö6983 were purchased from Calbiochem (San Diego, CA). Doxorubicin, etoposide, bisindolylmaleimide I (GF109203X, Bis I), rottlerin, propidium iodide (PI), 7-amino-4-methylcoumarin (AMC), methylthiazol tetrazolium, and RNase A were purchased from Sigma-Aldrich (St. Louis, MO). *N*-Acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) was obtained from Bachem (Bubendorf, Switzerland). All other chemicals were of analytical grade.

Cell Culture. MTLn3 rat mammary adenocarcinoma cells and MTLn3 cells overexpressing Bcl-2, or empty vector (Bcl-2 and Neo cells, respectively) were cultured as described previously (Huigsloot et al., 2002). Cells were exposed to doxorubicin for 1 h in Hanks' balanced salt solution and subsequently allowed to recover in α-MEM containing 2.5% fetal bovine serum for the indicated periods in the presence or absence of PKC inhibitors as indicated in the figure legends.

Transfection of Cells. For transient transfection, subconfluent cells were transfected with empty vector or pCO2-PKCα wild type (WT), pCO2-PKCα constitutively active or pKS1-PKCα dominant-negative PKCα constructs (kindly donated by Dr. P. J. Parker, Cancer Research UK, London Research Institute) in a 1:10 ratio with pGFP-histone 2B (GFP-H2B, kindly donated by Dr. J. Rohn, Leadd BV, Leiden, The Netherlands) using LipofectAMINE Plus reagent. Exposure to doxorubicin or etoposide occurred 24 h after transfection.

Determination of Cell Death. For cell cycle analysis, trypsinized and floating cells were pooled, washed with phosphate-buffered saline-EDTA, and fixated in 70% (v/v) ethanol (30 min at −20°C). After two washes, cells were incubated with phosphate-buffered saline-EDTA containing 50 μg/ml RNase A and 7.5 μM PI (45 min at room temperature) and subsequently analyzed by flow cytometry as described previously (Huigsloot et al., 2001). In addition, in some experiments, cell death was also analyzed by determining the percentage of Annexin V-positive/PI-positive cells [i.e., permeable cell membrane indicative of (secondary) necrosis] essentially the same as described previously (Huigsloot et al., 2001). Briefly, trypsinized and floating cells were pooled and centrifuged. Cells were then allowed to recover from trypsinization in complete medium (30 min at 37°C). Externalized phosphatidylserine was labeled (15 min at 0°C) with Alexa488-conjugated Annexin V in AV buffer (10 mM HEPES, 145 mM NaCl, 5 mM KCl, 1.0 mM $MgCl_2 \cdot 6H_2O$, 1.8 mM $CaCl_2 \cdot 2H_2O$; pH 7.4). Propidium iodide (2 μM) in AV buffer was added 1 min before analysis by flow cytometry on a FACScalibur (Becton Dickinson, San Jose, CA). Caspase-3-like activity was determined as described previously (Huigsloot et al., 2001). Briefly, cells were harvested, lysed, and incubated with 25 μM Ac-DEVD-AMC in assay buffer. The release of AMC was monitored (45 min at 37°C) in a fluorescence plate reader (PerkinElmer Instruments, Norwalk, CT). Free AMC was used as a standard, and caspase activity was expressed as picomoles of AMC per minute per milligram of protein.

Immunoblotting. Attached and floating cells were harvested, pooled, and immunoblotted as described previously (Huigsloot et al., 2002). Blots were probed for Bcl-2 (C-2; Santa Cruz Biotechnology, Santa Cruz, CA), active caspase-3 [CM-1, kindly provided by Dr. A. Srinivasan (Srinivasan et al., 1998)], GFP (BD Biosciences Clontech, Palo Alto, CA), PKCα, or PKCδ [m4 and δ14K, respectively, kindly provided by Dr. S. Jaken (Kiley et al., 1999a)]. Cytosolic fractions

were prepared and subsequently immunoblotted for cytochrome *c* as described previously (Huigsloot et al., 2002).

Soft Agar Colony Assay. MTLn3 cells were treated as described above, and 24 h after treatment, cells were trypsinized and 12,500 viable cells (trypan blue exclusion) were plated in soft agar as described previously (Huigsloot et al., 2002). methylthiazol tetrazolium-stained colonies were counted after 14 days. PKC inhibitors were added to the agar where indicated.

Statistical Analysis. Student's *t* test was used to determine whether there was a significant difference between two means ($p < 0.05$). When multiple means were compared, significance was determined by one-way analysis of variance ($p < 0.05$).

Results

Additive Effect of PKC Inhibition on Doxorubicin- or Etoposide-Induced Loss of Clonogenic Survival. Activation of PKC has been associated with cell proliferation and tumor formation; conversely, PKC inhibition reduces tumor cell growth in vivo. Therefore, we tested the effect of several PKC inhibitors on long-term survival and anchorage-independent growth capacity. The soft agar clonogenicity assay estimates both these properties. We used mammary adenocarcinoma MTLn3 cells, which like many epithelial tumorigenic cells can grow in an anchorage-independent fashion in soft agar (Kiley et al., 1999a). First, we characterized the effect of doxorubicin and etoposide on clonal survival. For this purpose, we exposed MTLn3 cells for 1 h to doxorubicin or etoposide, followed by recovery for 24 h to mimic clinically achieved peak plasma concentrations. To estimate the survival of tumor cells after treatment, equal numbers of viable cells were then plated in soft agar (based on trypan blue exclusion and microscopic evaluation) to reduce the bias from different numbers of dead cells in control versus treated cells. Both doxorubicin and etoposide strongly reduced the number of colonies of MTLn3 cells in soft agar in a concentration-dependent manner (Fig. 1). IC_{50} values for colony formation were 0.35 and 9.5 μM for doxorubicin and etoposide, respectively. The possibility existed that the decrease in colony formation was merely a direct consequence of the onset of cell death. To evaluate this, we next carefully analyzed the onset of apoptosis induced by both doxorubicin and etoposide (Fig. 2). Doxorubicin and etoposide caused a time- and concentration-dependent activation of caspase-3-like activity, indicative of activation of the apoptotic machinery (Fig. 2, A and D). This caspase activation was associated with the appearance of cells with hypoploid DNA content as determined by flow cytometric cell cycle analysis of cells with sub-G_0/G_1 DNA content (Fig. 2, B and E). Since eventually apoptotic cells may lose their plasma-membrane integrity and become necrotic, as a third measure of cell death, we also evaluated the appearance of necrosis using flow cytometric analysis of Annexin V and propidium iodide staining. The onset of necrosis (i.e., AV^+/PI^+ positive cells) followed the onset of apoptosis (caspase activation and sub-G_0/G_1 DNA content) in time for both doxorubicin and etoposide (Fig. 2, C and F). For etoposide, the inhibition of colony formation largely followed the onset of apoptosis. In contrast, importantly, no cell death, neither apoptosis nor necrosis, was observed at 0.5 μM doxorubicin up to 72 h. At this concentration, colony formation was inhibited by almost 80%. This indicates that the long-term inhibitory effects of doxorubicin on anchorage-independent growth are not directly correlated with the extent of apoptosis up to 72 h after treatment.

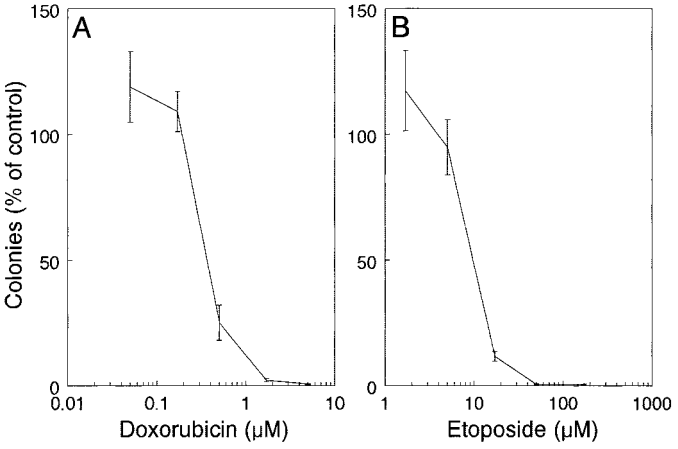

Fig. 1. Doxorubicin and etoposide induce a concentration-dependent decrease in colony formation. MTLn3 cells were exposed to doxorubicin (A) or etoposide (B) at the indicated concentrations for 1 h and subsequently allowed to recover for 24 h before plating in soft agar. Colony formation in agar is expressed as the percentage of vehicle-treated cells.

Next, we investigated the effect of various PKC inhibitors on doxorubicin- or etoposide-induced loss of anchorage-independent growth. Clonogenic growth was inhibited to about 50% by Gö6976 itself, which inhibits classic PKCs of which only PKCα is expressed in MTLn3 cells (Fig. 3) (Kiley et al., 1999a; Way et al., 2000). No effect was observed with Gö6983 and Bis I (GF109203X), which mainly inhibit classic PKCs and to some extent novel PKC isoenzymes (Way et al., 2000). An inhibitor of PKCδ, rottlerin, also had no effect on colony formation. Gö6976 further decreased colony formation up to 90% compared with doxorubicin or etoposide alone. Also, Gö6983 and Bis I potentiated the growth-inhibitory effect of doxorubicin, but these inhibitors had less effect when combined with etoposide. Inhibition of PKCδ using rottlerin had relatively little effect on either doxorubicin- or etoposide-induced loss of clonogenic survival. The combined data suggest that inhibition of classic PKC isoenzymes may be responsible for the reduction in clonogenic growth observed when cells are treated with doxorubicin or etoposide in combination with PKC inhibitors.

PKC Inhibitors Do Not Affect Doxorubicin- or Etoposide-Induced Apoptosis. PKC is involved in the regulation of apoptosis induced by a variety of stimuli. To test whether the reduction in colony formation by the PKC inhibitors was associated with an increase in apoptosis, we determined apoptosis after 24 h (i.e., the time at which the cells were plated for the colony assay). Treatment of MTLn3 cells with Gö6983 or Bis I alone did not induce apoptosis (Fig. 4). Gö6976 and rottlerin themselves slightly increased apoptosis, but this effect was not statistically significant. None of the inhibitors affected the extent of apoptosis induced by doxorubicin. This implies that apoptosis is not the major determinant for the long-term consequences of treatment with anticancer agents.

Bcl-2 Does Not Inhibit the Decrease in Long-Term Survival and Anchorage-Independent Growth Caused by Doxorubicin and Etoposide. Although Bcl-2 inhibits apoptosis, this may not affect the ability of anticancer agents to decrease long-term survival in the presence of increased levels of Bcl-2. Indeed, the effects of Bcl-2 on anchorage-independent growth are controversial. Bcl-2 overexpression

has been shown to be sufficient to allow anchorage-independent growth in bladder or thyroid tumor cells and to protect against γ irradiation-induced loss of clonogenic potential (Basolo et al., 1999; Miyake et al., 1999). On the other hand, in leukemic cells, overexpression of Bcl-2 or Bcl-x_L could not prevent Ara-C- or paclitaxel-induced loss of clonogenicity (Wang et al., 1999; Tang et al., 2000). To investigate whether Bcl-2 could prevent the loss of clonogenicity caused by doxorubicin and etoposide, we exposed Neo and Bcl-2 cells to a range of concentrations of these anticancer drugs. These cells have been created and characterized by us previously (Huigsloot et al., 2002). Bcl-2 overexpression by itself did not affect the ability to form colonies in soft agar (Fig. 5). Also, the loss of clonogenic growth upon exposure to both doxorubicin and etoposide was very similar for Neo and Bcl-2 cells (Fig. 5).

Next, we investigated whether the PKC inhibitors could potentiate the effect of doxorubicin and etoposide on clonogenic growth in Bcl-2 cells. Gö6976, Gö6983, and Bis I potentiated the inhibition of clonogenic growth by doxorubicin and etoposide to a similar extent in Neo cells as in parental MTLn3 cells (Figs. 3 and 6A). In MTLn3 cells with increased expression of Bcl-2, all three inhibitors further decreased the number of colonies formed upon exposure to doxorubicin to a similar extent as in Neo cells (Fig. 6B). In etoposide-treated cells, Gö6976 was also very potent in reducing the number of colonies, whereas Gö6983 and Bis I were less effective. This indicates that the combination of Gö6976 and doxorubicin

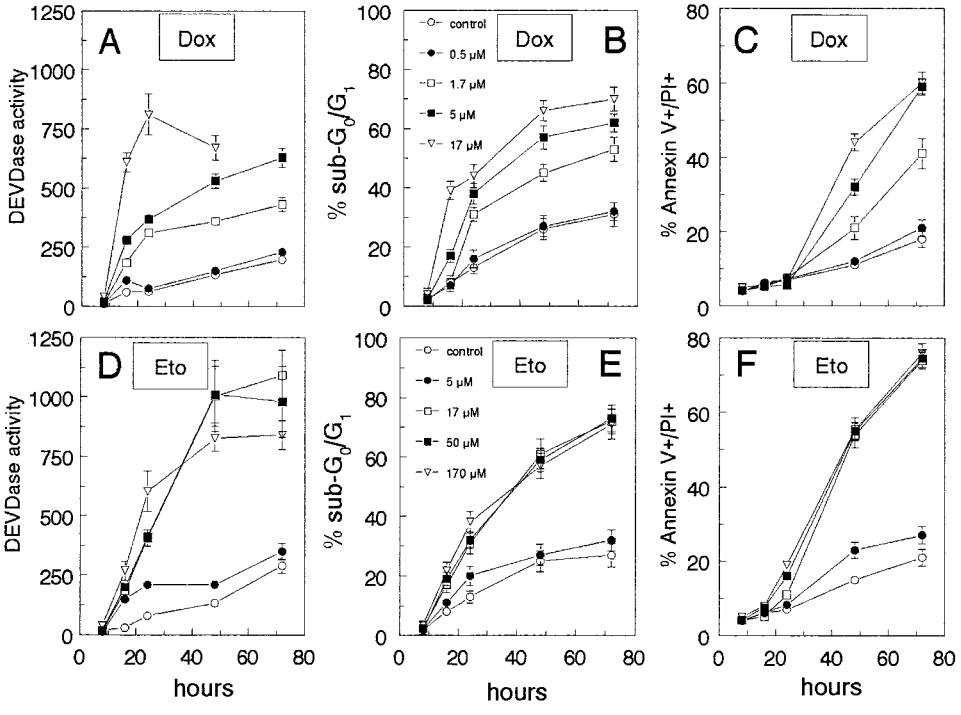

Fig. 2. Effect of doxorubicin (Dox) and etoposide (Eto) on cell viability of MTLn3 cells. MTLn3 cells were treated for 1 h with different concentrations of doxorubicin (A–C) or etoposide (D–F) followed by recovery in complete medium for various time periods. Cytotoxicity was evaluated by determination of caspase-3-like activity (A and D, DEVDase activity expressed as picomoles of AMC per minute per milligram of protein), flow cytometric analysis of the percentage of cells with sub-G_0/G_1-DNA content (B and E, i.e., apoptotic cells), and flow cytometric analysis of the percentage of cells that are positive for Annexin V-Alexa488/propidium ioide (C and F, AV^+/PI^+; i.e., necrotic cells). Data presented are the mean ± S.D. of three independent experiments (n = 3).

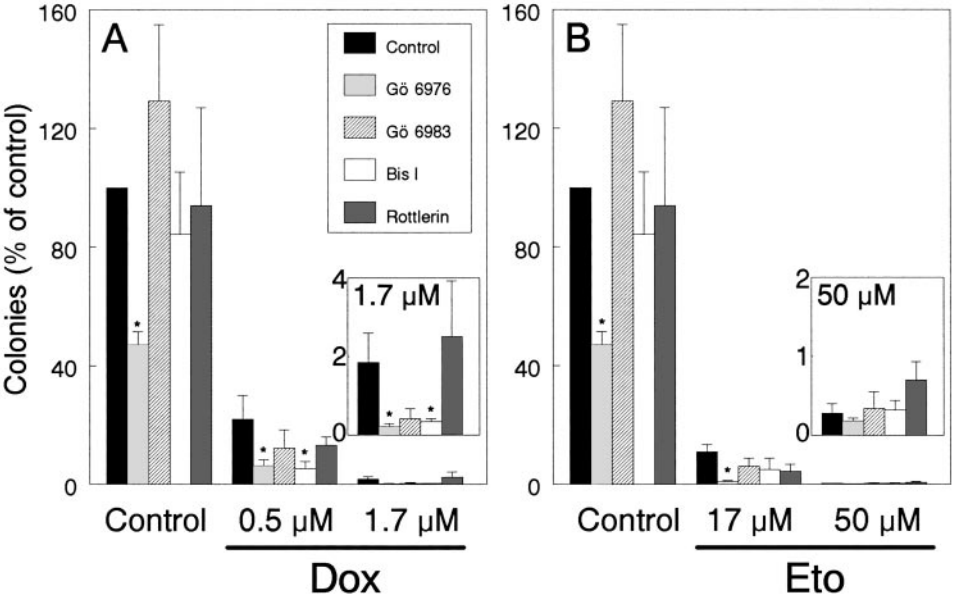

Fig. 3. Effect of PKC inhibitors on colony formation either alone or in combination with doxorubicin (Dox) or etoposide (Eto). MTLn3 cells were exposed to doxorubicin (A) or etoposide (B) as described in Fig. 1, except for the 24-h recovery phase when either vehicle, Gö6976 (500 nM), Gö6983 (500 nM), Bis I (250 nM), or rottlerin (5 μM) were added. Colony formation is expressed as the percentage of vehicle-treated cells. Data shown are the mean ± S.D. of two experiments in duplicate (n = 4). Asterisks indicate statistically significant differences between treatment control groups ($P < 0.05$).

may serve as an effective antitumor treatment even in tumors with high levels of Bcl-2.

Effect of Gö6976 on Doxorubicin-Induced Apoptosis in Neo and Bcl-2 Cells. Although inhibition of classic PKCs had no major effects on apoptosis in parental MTLn3 cells, it may affect the extent of apoptosis in Bcl-2 cells if combined with doxorubicin. Indeed, PKCα can phosphorylate Bcl-2 at Ser^{70}, a site that is essential for its antiapoptotic effect (Ruvolo et al., 1998). The fact that colony formation was as effectively inhibited by Gö6976 in Bcl-2 cells as in Neo cells also suggests that phosphorylation of Bcl-2 by PKCα may contribute to its protective effect. Inhibition of PKCα by coincubation with Gö6976 did not affect the extent of apoptosis in Neo cells (Fig. 7A). Bcl-2 overexpression inhibited apoptosis, as shown by DNA fragmentation, caspase activation, Western blotting for active caspase-3, PKCδ cleavage, and mitochondrial cytochrome *c* release (Fig. 7). Although Gö6976 did not affect caspase-3 cleavage, PKCδ cleavage and cytochrome *c* release, quantitative analysis of DNA fragmentation, and caspase activity revealed a slight, yet statistically significant increase in doxorubicin-induced caspase-3 (DEVDase) activity and DNA fragmentation in Bcl-2 cells. The more general PKC inhibitors Gö6983 and Bis I did not induce a significant increase in apoptosis caused by doxorubicin in Bcl-2 cells (Table 1). In conclusion, PKCα inhibition by Gö6976 only slightly affected the extent of apoptosis caused by doxorubicin in Bcl-2 cells.

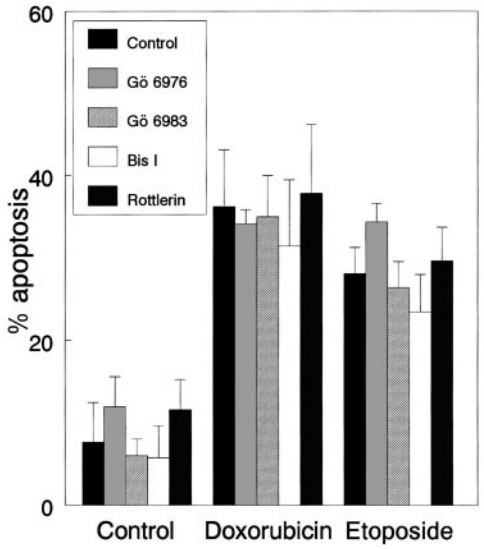

Fig. 4. Various PKC inhibitors do not induce apoptosis or affect anticancer drug-induced apoptosis. MTLn3 cells were treated with 17 μM doxorubicin or 50 μM etoposide for 1 h and subsequently allowed to recover for 24 h in the absence or presence of PKC inhibitors at the concentrations described in the legend for Fig. 2. Apoptosis was determined by flow cytometric analysis of DNA content. No statistically significant differences were observed between the absence or presence of PKC inhibitors.

Dominant-Negative PKCα Increased Apoptosis in Both Neo and Bcl-2 Cells. To test whether the effect of the PKC inhibitors on apoptosis in Bcl-2 cells is related to PKCα, we transfected Neo and Bcl-2 cells with WT, constitutively active, or dominant-negative PKCα. To distinguish transfected cells after fixation and during analysis of apoptosis by flow cytometry, cells were cotransfected with a GFP-histone 2B fusion construct that becomes integrated in nuclear DNA. Selection of GFP-positive cells was performed as indicated in Fig. 8A (top), and these cells were subsequently analyzed for DNA content (bottom). PKCα overexpression by the three constructs and GFP-histone 2B expression are shown by Western blotting (Fig. 8B). Similar expression of either protein was observed in Bcl-2 cells (data not shown). Overexpression of WT or constitutively active PKCα did not alter the percentage of apoptosis caused by doxorubicin in either Neo or Bcl-2 cells. However, dominant-negative PKCα did potentiate the apoptosis induction of doxorubicin in both Neo and Bcl-2 cells.

Gö6976 but Not Gö6983 or Bis I Cause Cell Cycle Arrest. The fact that Gö6976 itself inhibited clonogenic growth without inducing apoptosis led us to investigate whether changes in cell cycle progression may constitute the underlying mechanism. Gö6976 induced an accumulation in the G_2/M phase and to a lesser extent in the S phase in exponentially growing Neo cells after 24 h (Fig. 9A). This effect was even slightly stronger in Bcl-2 overexpressing cells (Fig. 9B). Upon treatment of Neo and Bcl-2 cells with Gö6983 or Bis I, no changes in cell cycle distribution were observed.

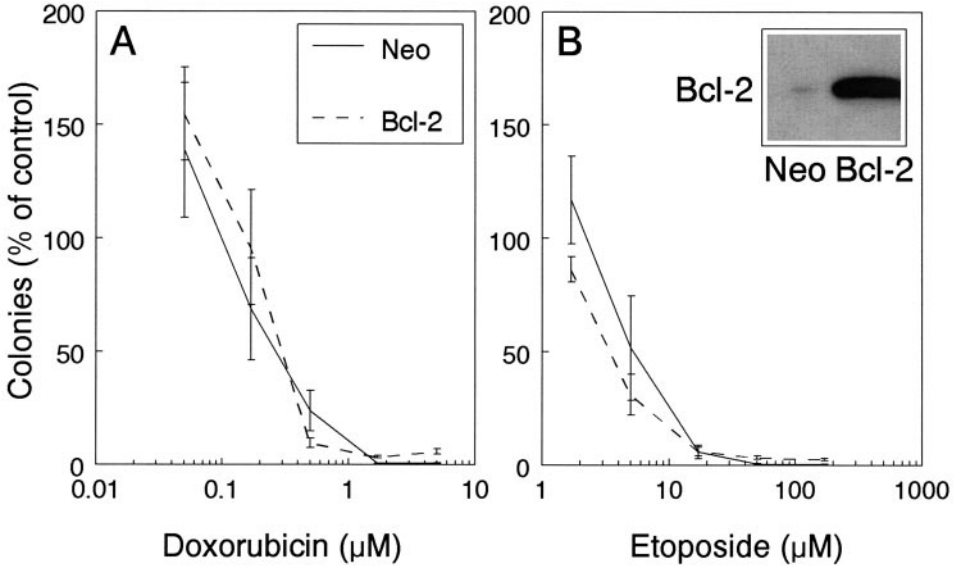

Fig. 5. Bcl-2 is unable to prevent the inhibition of colony formation by doxorubicin and etoposide. Both neomycin-resistant (Neo) and Bcl-2-overexpressing MTLn3 cells were treated and plated in soft agar as described in the legend for Fig. 1. Colony formation is expressed as the percentage of each vehicle-treated cell line. Untreated Bcl-2 cells grew slightly slower than neomycin-resistant cells (747 ± 61 and 862 ± 82 colonies/well, respectively).

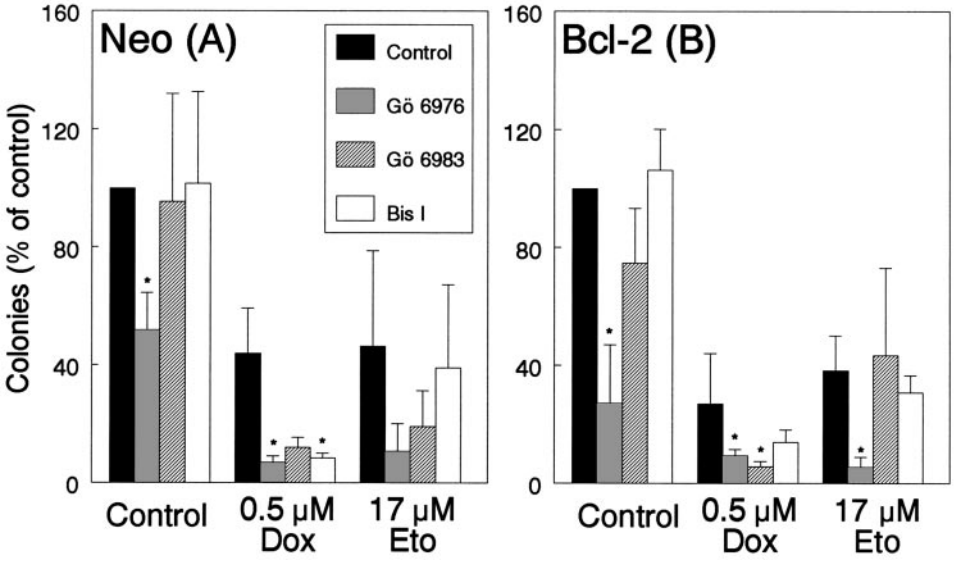

Fig. 6. Bcl-2 overexpression does not change the effect of PKC inhibitors on colony formation. Neo (A) and Bcl-2 (B) cells were treated as described in the legend for Fig. 2. Colony formation is expressed as the percentage of each vehicle-treated cell line. Data shown are the mean ± S.D. of two experiments in duplicate ($n = 4$). Asterisks indicate statistically significant differences between treatment control groups ($P < 0.05$).

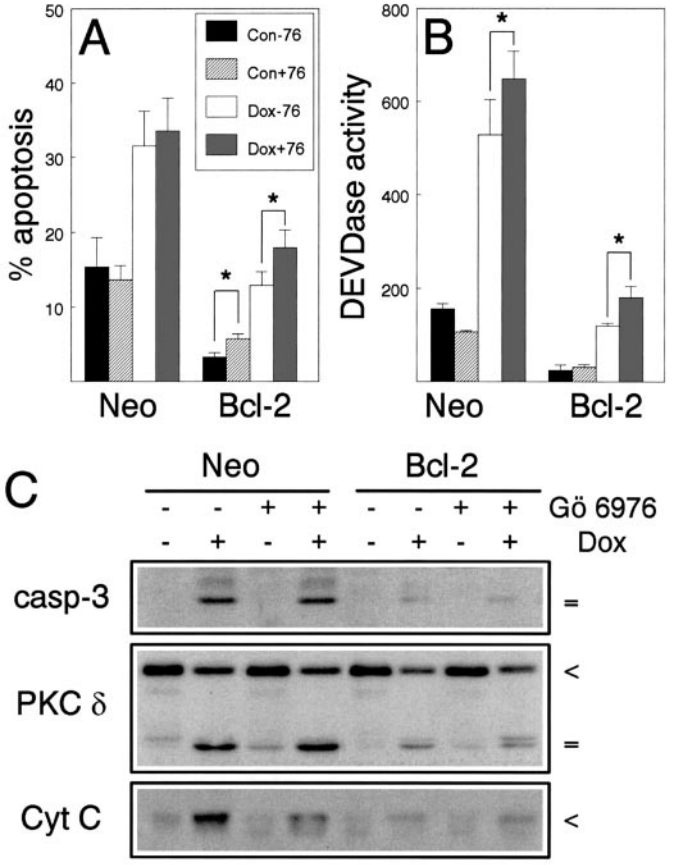

Fig. 7. Bcl-2 inhibits doxorubicin-induced apoptosis; Gö6976 slightly increased apoptosis in combination with doxorubicin in Bcl-2 cells. Neo and Bcl-2 cells were treated with vehicle (Con) or 17 μM doxorubicin (Dox) for 1 h and allowed to recover for 24 h in the absence (−) or presence (+) of 500 nM Gö6976 (76). Samples were taken to determine apoptosis (A), DEVDase activity (B, expressed as picomoles per minute per milligram of protein), and for Western blotting (C). The antibodies used were directed against active caspase-3, PKCδ, and cytochrome *c* (>, full-length proteins; =, cleavage products). Asterisks indicate statistically significant differences between the indicated treatment control groups ($P < 0.05$).

Since all three inhibitors potentiated the reduction in colony number caused by doxorubicin, we wanted to investigate whether the PKC inhibitors altered the cell cycle distribution in combination with doxorubicin. However, doxorubicin caused apoptosis, and thereby DNA fragmentation, as early as 16 h after exposure, which compromises cell cycle determination at late time points. Therefore, we determined the cell cycle changes at 6 h after exposure to PKC inhibitors in combination with doxorubicin (Table 2). Doxorubicin caused a decrease in the percentage of G_1 cells, accompanied by an increase in the S phase and a slight increase in the G_2 phase in both Neo and Bcl-2 cells. Treatment with the PKC inhibitors did not alter the cell cycle distribution compared with cells treated in the absence of inhibitors. This was the case for treatment with PKC inhibitors alone or in combination with doxorubicin. Therefore, the role of PKC inhibitor-induced cell cycle arrest in the decrease of colony formation in combination with doxorubicin remains to be elucidated.

Discussion

In the present study, we examined the role of PKC in loss of clonogenicity induced by the anticancer drugs doxorubicin and etoposide. The data indicate that PKCα is important in the regulation of cell growth and survival. First, inhibition of PKCα using Gö6976 as a single agent reduced anchorage-independent growth of MTLn3 cells. Second, in combination with doxorubicin or etoposide, inhibitors of PKCα further decreased colony number. Last, overexpression of Bcl-2 did not protect against the colony growth inhibitory effects of

TABLE 1
Effect of PKC inhibitors on apoptosis by doxorubicin
Neo and Bcl-2 cells were treated as described in the legend for Fig. 3. The percentage of apoptosis was determined 24 h after exposure by cell cycle analysis (percentage of sub-G_1/G_0 cells).

	Percentage of Apoptosis			
	Neo		Bcl-2	
	Control	Doxorubicin	Control	Doxorubicin
No inhibitor	15 ± 4	32 ± 5	3 ± 1	13 ± 2
Gö6983	9 ± 3	37 ± 6	2 ± 0	17 ± 3
Bis I	13 ± 4	33 ± 5	3 ± 0	17 ± 3

inhibitors of PKCα either alone or in combination with doxorubicin or etoposide. The inhibition of clonogenicity was not correlated with an increase in apoptosis. Together, these data indicate that a combination of inhibition of PKCα with conventional anticancer drugs is a promising approach for tumor treatment even if Bcl-2 is overexpressed.

MTLn3 cells predominantly express PKCα, δ, ϵ, and ζ but not PKCβ and η (Kiley et al., 1999a). The fact that PKCα is the only classic PKC isoenzyme expressed in these cells, indicates that this isoenzyme is most likely responsible for the inhibitory effect on colony formation of Gö6976, a specific inhibitor of PKCα and β (Way et al., 2000). Different effects were observed with Bis I and Gö6983. These inhibitors have different PKC isoenzyme specificity. Bis I inhibits classic isoenzymes and to some extent PKCϵ and δ. Gö6983 inhibits, besides classic PKCs, also PKCδ and ζ to a similar extent. Therefore, the difference in colony number upon exposure to the various inhibitors alone may be due to opposing roles of either PKCδ, ϵ, and/or ζ. Although all inhibitors further decreased colony number upon exposure to doxorubicin, Gö6976 was the only inhibitor that had a strong colony reducing effect in combination with etoposide. Both doxorubicin and etoposide induce double strand DNA breaks due to inhibition of topoisomerase II. However, doxorubicin is also known to cause formation of reactive oxygen species and to inhibit transcription. Therefore, the nature and extent of stress signals induced by these two compounds may be quite different. One of these signals may be PKCδ, which can be activated by etoposide; its overexpression increased etoposide-induced apoptosis (Reyland et al., 1999; Matassa et al., 2001). Whether PKCδ is activated by doxorubicin is not known. Gö6983 and Bis I differ from Gö6976 in that they both inhibit PKCδ besides PKCα. Thus, the fact that loss of colony formation by doxorubicin is potentiated by Gö6983 and Bis I (due to inhibition of PKCα, similar to Gö6976), but not in combination with etoposide (due to inhibition of both PKCα and δ), suggests that PKCδ is not involved in doxorubicin-induced stress signaling whereas it may be required for part of the inhibition of colony formation by etoposide.

Our results show that inhibition of PKCα using Gö6976 inhibits anchorage-independent growth in soft agar. Different kinds of experiments showed that inhibitors of classic PKCs, such as UCN-01 or using antisense oligonucleotides directed against PKCα, inhibited growth of glioma and liver cancer cells in monolayer culture (Pollack et al., 1996; Lin et al., 2000). Moreover, these inhibitors also inhibited tumor growth of glioma or liver and colon cancer cells in nude mice (Dean et al., 1996; Pollack et al., 1996; Yazaki et al., 1996). Here, we extend these findings and show that inhibition of PKCα can potentiate the inhibitory effect of doxorubicin and etoposide on anchorage-independent growth in soft agar in breast cancer cells. A combination of PKC modulators with anticancer drugs has only been used in leukemic cells. Thus, UCN-01, an inhibitor of both classic PKCs and CDK-1/2, could increase the cytotoxic effect of Ara-C in U937 cells (Tang et al., 2000). This was shown both at the level of apoptosis and colony formation in soft agar. These data once more indicate that inhibition of PKCα in combination with specific anticancer agents is promising for the treatment of cancers of different origin.

Tumor cell growth, both in vivo and in vitro, is determined by both proliferation rate and rate of cell death, including apoptosis. We found that the effect on apoptosis did not correlate with the long-term effects on clonogenicity in soft agar. Thus, Gö6976 alone or in combination with doxorubicin or etoposide did not significantly affect apoptosis, whereas it strongly reduced colony numbers in soft agar. This was also true for doxorubicin, which reduced colony formation at much lower concentrations than required for apoptosis. Moreover, overexpression of Bcl-2 effectively protected against doxorubicin-induced apoptosis, whereas it was unable to prevent loss of clonogenicity. This implies that the

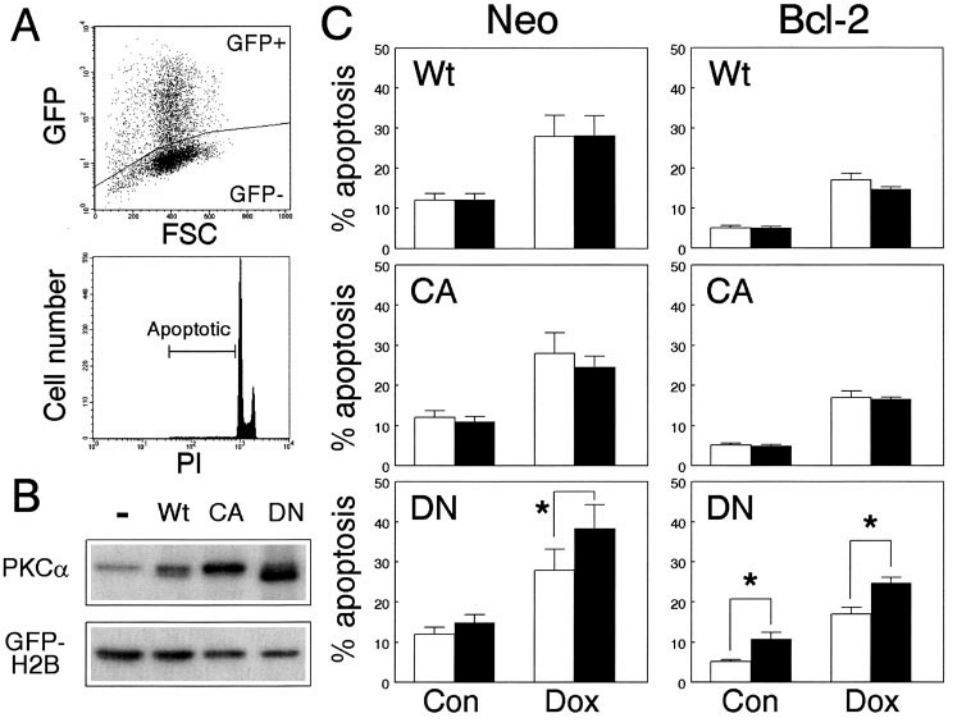

Fig. 8. Overexpression of dominant-negative PKCα mimics the effect of Gö6976 on the induction of apoptosis. Neo and Bcl-2 cells were transfected with constructs containing wild-type PKCα (Wt), constitutively active PKCα (CA), dominant-negative PKCα (DN), or no insert (−) in combination with GFP-H2B (ratio of 9:1). Cotransfection with GFP-H2B allows clear identification of transfected cells during flow cytometric analysis of DNA content (A). Expression of PKCα and GFP-H2B was determined using PKCα and GFP antibodies (B). At 24 h after transfection, cells were exposed to 17 μM doxorubicin or vehicle as described under *Materials and Methods* and after 24 h, DNA content was analyzed (C). White bars represent empty vector transfected cells and black bars represent cells transfected with the indicated constructs.

extrapolation of short-term effects on apoptosis in vitro to in vivo applications needs to be done with caution. Therefore, long-term growth assays such as anchorage-independent growth in soft agar, which also estimates some tumorigenicity parameters, may give a more accurate indication of treatment efficacy.

Bcl-2 is known to inhibit apoptosis induced by a variety of stimuli including doxorubicin and etoposide (Fig. 7) (Green and Reed, 1998; Gross et al., 1999; Huigsloot et al., 2002). Moreover, overexpression of Bcl-2 in leukemia cells has been associated with resistance to chemotherapy (Campos et al., 1993). In our hands, Bcl-2 overexpression was unable to protect against loss of anchorage-independent growth induced by doxorubicin or etoposide. Similar lack of long-term protection by Bcl-2 has been described for doxorubicin and etoposide in HeLa cells based on a monolayer culture clonogenicity assay (Lock and Stribinskiene, 1996; Elliott et al., 1998). Moreover, in leukemic cells Bcl-2 and Bcl-x_L were unable to prevent loss of colony formation in soft agar upon exposure to Ara-C or paclitaxel, respectively, whereas apoptosis was inhibited (Wang et al., 1999; Tang et al., 2000). In our Bcl-2 cells, the combination of either Gö6976 or dominant-negative PKCα with doxorubicin slightly increased the percentage of apoptosis and caspase activity compared with doxorubicin alone (Figs. 6, A and B, and 7C). This is another indication that the effects of Gö6976 may occur specifically through PKCα. Since we used transient transfection of the PKCα constructs with 25 to 35% transfection efficiency, we could not use these constructs in the soft agar assay. The reduction in protection by Bcl-2 upon PKCα inhibition may be due to reduced phosphorylation of Bcl-2 at Ser^{70}. This site has been shown to be phosphorylated by PKCα in vitro and its phosphorylation contributes to the antiapoptotic effect of Bcl-2 (Ito et al., 1997; Ruvolo et al., 1998). The fact that Bcl-2 cells were not protected from doxorubicin or etoposide in the anchorage-independent growth assay and that PKCα inhibition could still reduce the colony number further makes a combined treatment of PKCα inhibitors and cytostatics applicable regardless of the expression levels of Bcl-2.

One of the mechanisms that may be responsible for the colony growth inhibitory effects of Gö6976 is inhibition of cell cycle progression. Indeed, exposure to Gö6976 led to a G_2/M arrest at the time they were replated for the soft agar assay (Fig. 9). No changes in cell cycle distribution were observed with Bis I or Gö6983, which may explain the fact that only Gö6976 decreased colony number as a single agent. Although all three inhibitors potentiated the reduction in colony number caused by doxorubicin, we did not observe PKC-mediated differences in cell cycle distribution at 6 h after their combined treatment. Therefore, cell cycle changes may well be responsible for the reduction in colony formation by Gö6976 alone, whereas its role in the inhibition of clonogenic potential by all three PKC inhibitors in combination with doxorubicin remains to be elucidated.

In conclusion, inhibition of PKCα using Gö6976 alone inhibits anchorage-independent growth and even stronger in the presence of anticancer drugs. Although overexpression of Bcl-2 inhibited apoptosis induced by doxorubicin, Gö6976 could still decrease anchorage-independent growth either alone or in the presence of anticancer drugs to a similar extent as in Neo or MTLn3 cells. The colony inhibitory effects of Gö6976 are not associated with an increase in apoptosis and may be due to abrogation of normal cell cycling. Combination therapy may therefore be a promising strategy for (breast) cancer treatment, even if Bcl-2 is overexpressed.

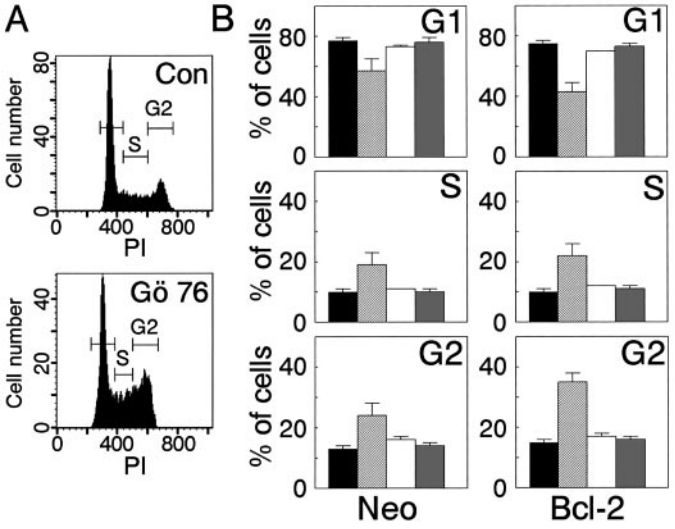

Fig. 9. Gö6976 induces G_2/M arrest in Neo and Bcl-2 cells. Neo and Bcl-2 cells were exposed to PKC inhibitors for 24 h at the concentrations indicated in the legend for Fig. 2, and the cell cycle distribution was determined as described under *Materials and Methods*. Black bars indicate cells treated in the absence of PKC inhibitors; hatched, white, and gray bars indicate cells treated with Gö6976, Gö6983, or bisindolylmaleimide I, respectively. Representative cell cycle distributions of Neo cells are shown in A (top, vehicle; bottom, Gö6976). Cell cycle distribution was calculated according to the markers in A and plotted in B.

TABLE 2

Cell cycle distribution 6 h after exposure to doxorubicin

Neo cells were treated for 1 h with vehicle or 17 μM doxorubicin and subsequently allowed to recover for 6 h. The percentage of cells in each cell cycle phase was determined by cell cycle analysis as described under *Materials and Methods*.

	Cell Cycle Distribution (Percentage of Cells)		
	G_1	S	G_2
Control			
−Inhibitor	61 ± 2	12 ± 1	27 ± 1
+Gö6976	62 ± 1	11 ± 1	27 ± 0
17 μM Doxorubicin			
−Inhibitor	50 ± 3	22 ± 1	29 ± 2
+Gö6976	53 ± 1	18 ± 0	29 ± 2

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