

# Novel Extranuclear-targeted Anthracyclines Override the Antiapoptotic Functions of Bcl-2 and Target Protein Kinase C Pathways to Induce Apoptosis<sup>1</sup>

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## Abstract

**Bcl-2 inhibits apoptosis induced by numerous antitumor drugs, including doxorubicin and daunorubicin and is, thus, a major impediment to successful cancer chemotherapy. Here, we report the ability of a novel family of nonnuclear targeted anthracyclines to induce rapid apoptosis in cells despite Bcl-2 or Bcl-X<sub>L</sub> expression. Typified by *N*-benzyladriamycin-14-valerate (AD 198) and *N*-benzyladriamycin-14-pivalate (AD 445), this family of compounds binds to the C1 regulatory domain of protein kinase C (PKC), competitively inhibits phorbol ester binding in cell-free studies, and induces PKC translocation in intact cells. PKC- $\delta$  has an established role as a pro-apoptotic protein through the association of the holoenzyme or catalytic fragment with mitochondria. In proliferating 32D.3 myeloid cells, or in 32D.3 cells engineered to overexpress Bcl-2, substantial levels of PKC- $\delta$  are associated with mitochondria. However, after a 1-h exposure to 5  $\mu$ M AD 198, cytochrome *c* release, caspase-3 activation, poly(ADP-ribose) polymerase (PARP) cleavage, PKC- $\delta$  cleavage, and DNA fragmentation are observed. Pretreatment of 32D.3 cells with the selective PKC- $\delta$  inhibitor, rottlerin, but not the general PKC inhibitor, GF 109203X, or PKC- $\alpha$  and - $\beta$  inhibitor, Gö 6976, delayed the 50% cell kill to >24 h for control and Bcl-2 overexpressing 32D.3 cells treated with 5  $\mu$ M AD 198. Rottlerin delayed PKC- $\delta$  and PARP cleavage to >20 h post-drug exposure and also delayed mitochondrial membrane depolarization. In contrast, the pan-caspase inhibitor Z-Val-Ala-Asp-CH<sub>2</sub>F blocked PKC- $\delta$  and PARP cleavage, but not mitochondrial membrane**

**depolarization. These results suggest that AD 198 induces mitochondrial-dependent apoptosis in 32D.3 cells by activating PKC- $\delta$  holoenzyme on mitochondria, which, in turn, overrides the antiapoptotic effects of Bcl-2.**

## Introduction

Apoptosis is a morphologically unique mechanism of cell death characterized by three phases: a heterogeneous initiation phase in which a variety of cellular signaling pathways respond to the presence of both exogenous and endogenous apoptogenic factors; a decision phase in which the cell commits to a specific mechanism of cell death; and an execution phase in which the cell is enzymatically dismembered (1). In type II mammalian cells, as defined by Scaffidi *et al.* (2), changes in mitochondrial integrity play a pivotal role in the regulation of apoptotic progression (reviewed in Ref. 3). Pro-apoptotic signaling molecules, accumulating in the cytoplasm during the initiation phase, trigger the formation of mitochondrial permeability transition pore complexes that permit the release of apoptogenic components, such as cytochrome *c* and apoptosis-inducing factor, from the intermembrane space into the cytosol. These factors catalyze the activation of caspases-9, -6, and -3, leading to caspase-mediated activation of pro-apoptotic enzymes (DFF40 endonuclease, PKC<sup>3</sup>- $\delta$ ), inactivation of repair and signaling enzymes (PARP, DNA-dependent protein kinase, and protein phosphatases), cleavage of structural proteins (lamin, fodrin, and actin), and step-wise endonucleolytic fragmentation of DNA (reviewed in Ref. 4). Consequently, control of mitochondrial integrity is a critical component of apoptotic regulation. On initiation of apoptosis, members of the Bcl-2 family of proteins can interact with mitochondria to function as either pro-apoptotic (Bax, Bad, Bcl-X<sub>S</sub>, Bid) or antiapoptotic (Bcl-2, Bcl-X<sub>L</sub>, A1, Mcl-1) factors and facilitate or block structural and functional mitochondrial changes that are required for the stimulation of the effector events of apoptosis (3, 5). Of particular clinical concern is the expression of antiapoptotic Bcl-2 proteins in a wide variety of tumors that effectively block apoptosis induced by virtually all chemotherapeutic

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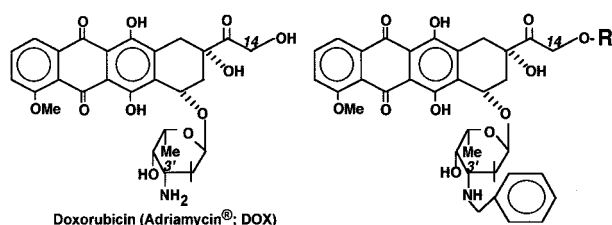
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<sup>3</sup> The abbreviations used are: PKC, protein kinase C; AD 198, *N*-benzyladriamycin-14-valerate; AD 445, *N*-benzyladriamycin-14-pivalate; AD 442, *N*-benzyladriamycin-14-acetate; AD 443, *N*-benzyladriamycin-14-propionate; AD 444, *N*-benzyladriamycin-14-butyrate; DOX, doxorubicin; topo II, DNA topoisomerase II; PDBu, phorbol 12,13-dibutyrate; AD 288, *N*-benzyladriamycin; TNF, tumor necrosis factor; IL-3, interleukin 3; GF, GF 109203X; Z-VAD-fmk, Z-Val-Ala-Asp-CH<sub>2</sub>F; PARP, poly(ADP-ribose) polymerase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Tdt, terminal deoxynucleotidyl transferase; TUNEL, Tdt-mediated nick end labeling; RIPA, radioimmunoprecipitation assay; TDN, *trans*-dominant negative; HRP, horseradish peroxidase.

agents as well as by radiation, hypoxia, nutrient deprivation, and increased cytosolic  $\text{Ca}^{2+}$  levels (6–8). Consequently, improved chemotherapeutic management of Bcl-2-expressing tumor cells would require agents that either inactivate or circumvent the antiapoptotic functions of Bcl-2 proteins. However, efforts to design therapies that circumvent Bcl-2 have met with only limited success (9–12).

The ability of Bcl-2 to suppress apoptosis is modulated, at least in part, by serine phosphorylation at multiple sites on the protein. Ito *et al.* (13) reported that phosphorylation results in activation of Bcl-2 and suppression of apoptosis. Contrasting studies suggest that hyperphosphorylation of Bcl-2 inactivates its functions and leads to apoptosis (14–16). This difference may be cell type-specific or may be explained by the observation that activation at one site is necessary for further phosphorylation at additional sites, which inactivates the protein (13–15). The mechanism by which Bcl-2 phosphorylation is modulated is not yet clear, but appears to be caused by multiple kinases, including PKC, p38 mitogen-activated protein kinases (p38 MAPKs), JNK-1, and Raf-1 kinase (17–21). Consistent with the role of PKC in the control of Bcl-2 activity are observations that suggest that the PKC inhibitor staurosporine and the PKC activator bryostatin-1 modulate Bcl-2 phosphorylation status and its antiapoptotic activity (17, 20, 22).

The novel agent AD 198 is the lead compound of a new class of extranuclear-targeted acylantracyclines that are functionally distinct from the DNA-binding anthracyclines such as DOX or daunorubicin. Unlike DOX, which stabilizes the topo II/DNA cleavable complex to induce double-stranded DNA cleavage, AD 198 instead binds to the C1 regulatory domain of conventional and novel PKC isozymes in a manner that competitively inhibits phorbol ester binding (23). Molecular modeling studies show that within AD 198, the three-dimensional structure of the valerate group in combination with portions of the chromophore A ring form the pharmacophore found in diacylglycerol (DAG) and phorbol esters that allows binding to the C1 regulatory domain of PKC. In an accompanying report (24), we show that AD 198 displaces the radiolabeled phorbol ester, [ $^3\text{H}$ ]-PDBu, from its C1 binding site on PKC- $\delta$  holoenzyme and isolated PKC- $\delta$  C1 domain in a dose-dependent manner. The extent of [ $^3\text{H}$ ]-PDBu displacement correlates with increasing 14-O-acyl chain length from 0 to 5 carbons, with the 4- and 5-carbon chains having the maximal effect on displacement. This phenomenon correlates with a dose-dependent reduction in phorbol ester-induced PKC activity in cell-free assays but rapid PKC translocation in intact cells (23, 24). Although AD 198 is biotransformed through enzymatic and nonenzymatic hydrolysis to AD 288, a catalytic inhibitor of topo II (25), AD 198 is not a pro-drug, but rather induces rapid apoptosis in the absence of significant biotransformation (24). In addition, AD 198 rapidly accumulates in the cytoplasm and is able to circumvent multiple mechanisms of cellular drug resistance, including those mediated by overexpression of the multidrug transporters P-glycoprotein and multidrug resistance protein (MRP), and by reduced topo II activity (26–29). As a ligand for PKC, the cytotoxic mechanism of AD 198 may involve the modulation of PKC-mediated signaling. In addition to the role



Doxorubicin (Adriamycin®; DOX)

Name	Code Number	R
<i>N</i> -Benzyladriamycin	AD 288	H
<i>N</i> -Benzyladriamycin-14-acetate	AD 442	
<i>N</i> -Benzyladriamycin-14-propionate	AD 443	
<i>N</i> -Benzyladriamycin-14-butyrate	AD 444	
<i>N</i> -Benzyladriamycin-14-valerate	AD 198	
<i>N</i> -Benzyladriamycin-14-pivalate	AD 445	

Fig. 1. Chemical structures of relevant anthracyclines.

of the PKC protein family in regulating cell growth and differentiation, various holoisozymes, including PKC- $\alpha$  (30), PKC- $\delta$  (31–34), and PKC- $\epsilon$  (34) have apoptotic activity. PKC- $\delta$  translocation to mitochondria alters mitochondrial membrane potential and induces cytochrome *c* release (31, 32), whereas cleavage of PKC- $\delta$  and - $\epsilon$  by caspase-3 releases the catalytic subunit and, likewise, triggers mitochondrial release of cytochrome *c* and thus contributes to the effector stage of apoptosis (35, 36).

In this report, we demonstrate that AD 198 induces apoptosis in a manner that is unaffected by Bcl-2 expression and that correlates with PKC activation. Our findings suggest that AD 198 rapidly induces PKC- $\delta$  activity to trigger mitochondrial-dependent apoptosis even in cells that express high levels of Bcl-2 or Bcl-X<sub>L</sub>.

## Materials and Methods

**Cell Culture and Reagents.** IL-3-dependent 32D.3 murine myeloid cell lines transfected with either empty SFFV expression vector (37) or SFFV-Bcl-2 (32D.3/Bcl-2), were described previously (38) and were maintained in RPMI 1640 with L-glutamine (Atlanta Biologicals, Norcross, GA) supplemented with 10% fetal bovine serum (Atlanta Biologicals) and 40 units/ml IL-3 (Sigma Chemicals, St. Louis, MO; Ref. 39). MCF-7 wild-type cells were obtained from the American Type Culture Collection (Manassas, VA). MCF-7 variant M cells, selected for resistance to TNF- $\alpha$  (40), were the generous gift of Dr. Steven Hill (Tulane University, New Orleans, LA). Both cell lines were maintained as described previously (41). DOX was obtained from Sigma Chemicals, and AD 288, AD 198, AD 442, AD 443, AD 444, and AD 445 hydrochloride salts were prepared in this laboratory according to previously described procedures (Ref. 29; Fig. 1). Rottlerin, GF, Gö 6976, and Z-VAD-fmk were obtained from Calbiochem (La Jolla, CA). All of the drugs were dissolved in DMSO. Final DMSO concentration (1%) used in drug treatments was not cytotoxic.

**Drug Treatment and Cell Viability Determination.** 32D.3 cells, at a density of  $5 \times 10^5$ /ml, were exposed to the  $IC_{90}$  concentrations ( $5 \mu\text{M}$ ; drug concentration required to kill 90% of 32D.3 cells 72 h after a 1-h drug treatment) of DOX, AD 288, AD 442, AD 443, AD 444, AD 198, or AD 445 in medium for 1 h at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$ -humidified environment. Control cells were treated with 1% DMSO for 1 h. After drug treatment, cells were washed twice with PBS and incubated further in drug-free media. At multiple times between 6 and 72 h,  $75\text{-}\mu\text{l}$  aliquots of cell suspension were combined with  $25 \mu\text{l}$  of trypan blue (Life Technologies, Inc., Rockville, MD) and assayed microscopically for cellular dye exclusion. Alternatively, cells were treated continuously with either  $10 \mu\text{M}$  rottlerin,  $2 \mu\text{M}$  GF,  $1 \mu\text{M}$  Gö 6976, or 1.0% DMSO, beginning 2 h before anthracycline exposure as described above, or were pretreated with  $200 \mu\text{M}$  Z-VAD-fmk for 1 h, followed by continuous drug exposure to  $5 \mu\text{M}$  AD 198. Cell viability for MCF-7 cells lines was determined using the MTT assay (42).

**Analysis of DNA Fragmentation.** DNA fragmentation of apoptotic cells was monitored by the TUNEL assay as described by the supplier (Promega, Madison, WI). Briefly, cells were treated with  $5 \mu\text{M}$  drug for 1 h, washed twice in warm PBS, incubated in drug-free medium for the indicated times, then fixed in 4% methanol-free formaldehyde in PBS (pH 7.4) for 25 min at  $4^\circ\text{C}$ . Cells were permeabilized in 0.2% Triton X-100 in PBS for 5 min on ice. Tdt labeling of 3' ends of DNA strand breaks in apoptotic cells with fluorescein-12-dUTP was performed with an apoptosis detection system. After end-labeling, cells were then washed with PBS containing 0.1% Triton X-100 and 5 mg/ml BSA. All of the cells were stained with  $1 \mu\text{g/ml}$  propidium iodide for 15 min. Green and red fluorescence emissions were observed microscopically (Olympus model BX60) using 520 nm and  $>620$  nm filters, respectively. DNA fragmentation into 50–300 kb was detected by pulsed field agarose gel electrophoresis using a CHEF gel system (Bio-Rad Laboratories, Richmond, CA). Briefly, cells were exposed to  $5 \mu\text{M}$  AD 198 for 1 h, followed by incubation in drug-free medium for 1, 2, or 4 h. Control cells were exposed to 1% DMSO for 1 h, followed by incubation in drug-free media for 4 h. Cells were washed twice in cold PBS and resuspended in 1% low-melting-point agarose with 0.125 M EDTA. Samples were loaded into plug-casting blocks and incubated at  $4^\circ\text{C}$  for 30 min. The agarose plugs were incubated in 0.5 M EDTA (pH 8.0), 1 mM Tris-HCl (pH 7.5), and 2 mM  $\beta$ -mercaptoethanol at  $37^\circ\text{C}$  for 24 h, followed by incubation in 1% laurylsarcosine, 10 mM Tris-HCl (pH 7.5), and 0.5 M EDTA with proteinase K for 24 h at  $50^\circ\text{C}$ . The plugs were then incubated for two consecutive 24-h periods in 50 mM EDTA (pH 8.0) at room temperature. The DNA was resolved by pulse-field gel electrophoresis in 1.2% agarose at 150 V for 48 h at  $14^\circ\text{C}$  with a switch time of 5.4–54 s. The gel was stained with ethidium bromide and photographed.

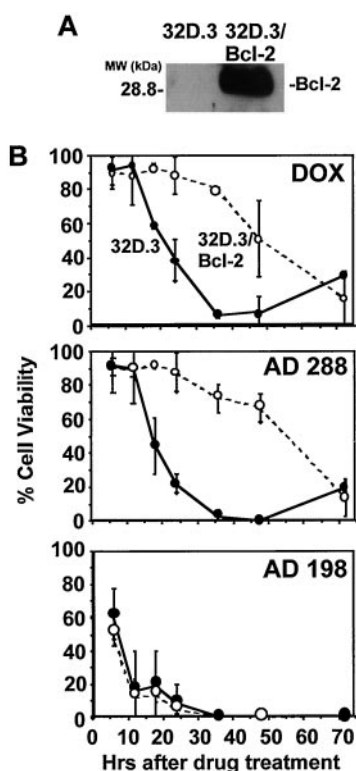
**Protein Extraction for Bcl-2, Cytochrome c, PARP, and Caspase-3 and Analyses.** Analysis of Bcl-2 levels was performed on whole-cell extracts. Cells were exposed to  $5 \mu\text{M}$  AD 198 for 1 h, washed twice in warm PBS, and incubated in drug-free medium. Cells were then washed in cold PBS, and resuspended in SDS-PAGE sample buffer (43), heated at  $95^\circ\text{C}$  for 5 min, and subjected to SDS-PAGE in 12% poly-

acrylamide. For cytochrome c release into cytosol, cells were treated with a drug as described above. At the times indicated, cells were washed in cold PBS, resuspended in mitochondrial isolation buffer (250 mM sucrose, 20 mM HEPES- $\text{KO}_4$  (pH 7.4), 1.5 mM  $\text{MgCl}_2$ , 10 mM KCl, 1 mM EDTA, with 1 mM PMSF,  $20 \mu\text{M}$  leupeptin,  $50 \mu\text{g/ml}$  pepstatin A,  $50 \mu\text{g/ml}$  aprotinin, and 2 mM DTT to inhibit proteolytic activity), and incubated on ice for 20 min. Cells were lysed with a Dounce homogenizer, and the lysate was subjected to centrifugation at  $14,200 \times g$  for 15 min at  $4^\circ\text{C}$ . The resulting supernatant containing the cytosolic fraction was lyophilized, then suspended in RIPA buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% NP40, and 0.5% sodium deoxycholate]. After protein determination of the samples,  $100 \mu\text{g}$  of protein were resolved by SDS-PAGE in 15% polyacrylamide. For caspase-3 and PARP analysis, cells were exposed for 1 h to  $5 \mu\text{M}$  AD 198 or 1% DMSO, followed by incubation in drug-free medium. At the times indicated, cells were washed twice in cold PBS, resuspended in RIPA buffer and incubated on ice for 30 min. The lysate was centrifuged at  $14,200 \times g$  for 10 min at  $4^\circ\text{C}$  to pellet heavy membrane and DNA. After protein determination of the samples, protein samples ( $125 \mu\text{g}$ ) were resolved by SDS-PAGE in 12% polyacrylamide.

**Cell Fractionation for PKC- $\alpha$  and PKC- $\delta$  Translocation Analyses.** Translocation analyses were performed as described previously (44) with minor modifications. Briefly, cell aliquots were washed twice with cold PBS, resuspended in lysis buffer A [25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM  $\text{MgCl}_2$ , 1 mM EGTA, and 1 mM EDTA with protease inhibitors described above] and lysed by sonication. Aliquots of cell lysates were set aside for total protein samples, and the remaining lysate was subjected to centrifugation at  $96,000 \times g$  for 1 h at  $4^\circ\text{C}$  to separate the cytosolic fraction from the pelleted membrane and detergent-insoluble material. The pellet was resuspended in lysis buffer B [25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM  $\text{MgCl}_2$ , 1 mM EGTA, 1 mM EDTA, and 1% TX-100 with protease inhibitors] and centrifuged at  $14,200 \times g$  for 15 min at  $4^\circ\text{C}$  to separate the membrane from detergent insoluble fraction. The insoluble fraction was resuspended in  $2\times$  concentrated SDS-PAGE sample buffer without glycerol,  $\beta$ -mercaptoethanol, or dye, and heated to  $95^\circ\text{C}$  to dissolve the pellet. Small aliquots were taken from each fraction and total protein and were set aside for protein determination as described above. All of the samples were stored at  $-20^\circ\text{C}$ .

**Immunoblot Analysis.** Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The filters were blocked by incubating for 30 min in 5% nonfat dry milk in PBS with 0.05% Tween 20 (5% MLK/PBST). The filters were then incubated with primary antibody in 5% MLK/PBST, washed three times for 5 min each with PBST, incubated with secondary antibody, and washed three times for 5 min each with PBST. Immunoreactive bands were detected by chemiluminescence (Pierce, Rockford, IL). For details of individual antibodies, see Fig. legends.

**Mitochondrial Membrane Depolarization.** 32D.3 cells were treated with rottlerin or Z-VAD-fmk and AD 198 as described above. Cells were then collected and washed

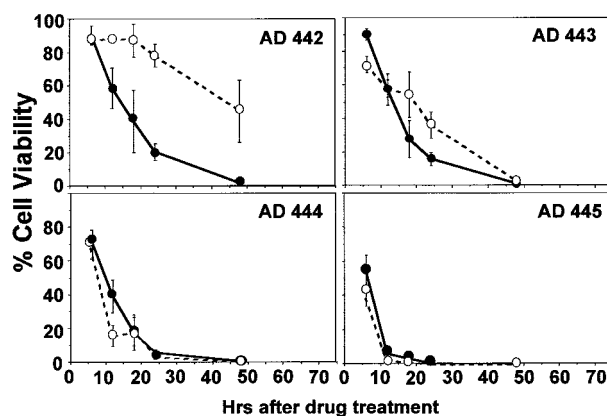


**Fig. 2.** AD 198 cytotoxicity is unaffected by Bcl-2 expression in 32D.3 myeloid cells. **A**, 40  $\mu\text{g}$  of protein from whole-cell lysates of 32D.3 cells and 32D.3/Bcl-2 were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with anti-Bcl-2 rabbit polyclonal antibody (1:000; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h, followed by goat antirabbit-HRP conjugate (1:3000; Upstate Biotechnology, Lake Placid, NY) for 1 h. Proteins were detected by chemiluminescence. **B**, cells were suspended in supplemented RPMI 1640 at a density of  $5 \times 10^5$  cells/ml and exposed to 5  $\mu\text{M}$  drug for 1 h at 37°C. Cells were resuspended in fresh, drug-free medium and incubated at 37°C for up to 72 h. At the times indicated, aliquots of cells were withdrawn and stained with trypan blue. Viable cells were scored based on the exclusion of stain and gross morphological appearance. Each datum point, the mean ( $\pm$ SE) of at least three independent determinations, each consisting of 300–500 cells/count. ●, 32D.3 cells; ○, 32D.3/Bcl-2 cells.

twice in warm PBS and prepared for staining with 5,5',6,6',tetrachloro-1,1', 3,3'-tetraethylbenzimidazolyl carbocyanin iodide (DePsipher; Trevigen, Inc., Gaithersburg, MD) according to the manufacturer's instructions. Cellular red fluorescence patterns were observed by fluorescence microscopy as described above.

## Results

Murine IL-3-dependent myeloid 32D.3 cells expressing high levels of human Bcl-2 (32D.3/Bcl-2) or transfected with empty vector (32D.3; Fig. 2A; Ref. 38) were treated with the  $\text{IC}_{90}$  drug concentration, as calculated for 32D.3 cells, of DOX, AD 288, and AD 198. Cell kill was monitored for 72 h by trypan blue staining after a 1-h drug exposure (Fig. 2B). In 32D.3 cells, DOX and AD 288 treatments resulted in 90% cell kill within 36 h of drug exposure. However, the expression of Bcl-2 in 32D.3/Bcl-2 cells delayed 90% cell kill for both DOX and AD 288 until >72 h. In contrast, 90% cell kill by AD 198

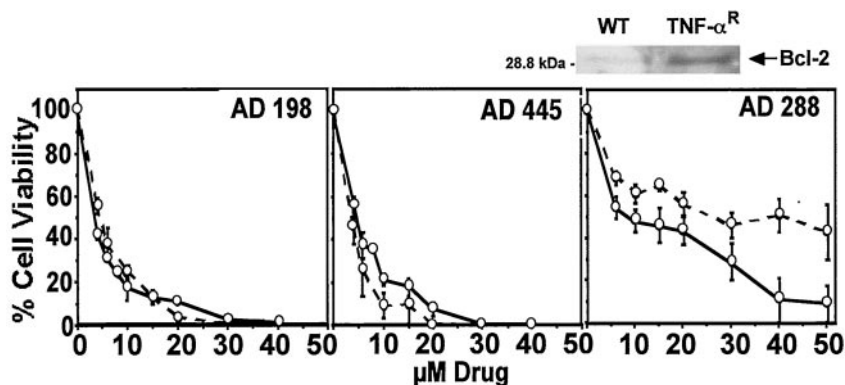


**Fig. 3.** Circumvention of Bcl-2 inhibition of cytotoxicity correlates with increasing 14-O-acyl chain length. 32D.3 and 32D.3/Bcl-2 cells were treated with 5  $\mu\text{M}$  each compound indicated, as described in Fig. 2. Each datum point, the mean ( $\pm$ SE) of at least three independent determinations, each consisting of 300–500 cells/count. ●, 32D.3 cells; ○, 32D.3/Bcl-2 cells.

was achieved  $\sim$ 12 h after drug exposure in both 32D.3 and 32D.3/Bcl-2 cells. Identical results were observed in a 32D.3 cell line engineered to express high levels of murine Bcl-X<sub>L</sub> (data not shown). Although the presence of the *N*-benzyl moiety alone (i.e., AD 288) was insufficient to bypass the antiapoptotic effects of Bcl-2, the 14-O-valerate side chain appears to be essential. Because the 14-O-valerate moiety confers PKC modulatory activity that decreases with decreasing acyl chain length (24), it was next determined whether PKC modulatory activity correlated with cytotoxicity against Bcl-2-expressing cells. To address this issue, a series of *N*-benzylated 14-O-acyl substituted anthracyclines (AD 442, AD 443, AD 444, AD 198, and AD 445; Fig. 1), developed previously in our laboratories, was used (29). The cytotoxicity of AD 442, possessing a 14-O-acetate moiety, was impaired by Bcl-2 expression in 32D.3/Bcl-2 cells to an extent similar to that with DOX and AD 288. However, elongation of the 14-O-acyl substituent chain length progressively reduced the resistance of Bcl-2-overexpressing cells (Fig. 3). Like AD 198, the 5-carbon pivalate substitution (AD 445) resulted in rapid and complete circumvention of Bcl-2 antiapoptotic activity. In addition, control 32D.3 cell kill was progressively more rapid as 14-O-acyl chain length was increased. These results indicate that lengthening the 14-O-acyl chain on *N*-benzyladriamycin results in increasingly rapid and more complete circumvention of the antiapoptotic functions of Bcl-2. The cytotoxicities of AD 198 and AD 445 were also compared with those of AD 288 in wild-type MCF-7 breast carcinoma cells and a TNF- $\alpha$ -resistant MCF-7 cell line expressing elevated levels of endogenous Bcl-2 (Fig. 4). MTT cytotoxicity assays revealed that the Bcl-2-expressing cells had an increased  $\text{IC}_{50}$  value of AD 288 of 25  $\mu\text{M}$  versus 8  $\mu\text{M}$  for wild-type cells. However, the  $\text{IC}_{50}$  values of both AD 198 and AD 445 were unaffected by Bcl-2 expression and were  $\sim$ 4  $\mu\text{M}$  for both drugs in both cell lines.

Given the rapid rate of cell kill by AD 198 and AD 445 and the lack of inhibition by Bcl-2 expression, it was important to

**Fig. 4.** AD 198 and AD 445 cytotoxicities are unaffected by Bcl-2 expression in MCF-7 cells. Bcl-2 detection in whole-cell lysates were performed as described in Fig. 2A. Attached cells in DMEM/10% FCS/20  $\mu$ M insulin at a density of  $1-2 \times 10^4$  cells/ml were exposed to the indicated concentrations of drug for 1 h at 37°C. Cells were then washed twice in warm PBS and incubated at 37°C for 48 h. Viable cells were quantified by the addition of MTT and subsequent spectrophotometric analysis. Each datum point, at least three independent determinations. —○—, wild-type (WT); —○—, TNF- $\alpha$  resistant (TNF- $\alpha^R$ ).



confirm that AD 198 cell kill was indeed apoptotic. Drug-treated 32D.3 cells were assessed for DNA fragmentation by both TUNEL assay and pulsed-field gel electrophoresis. DNA fragmentation, as indicated by 3'-end labeling with fluorescein-12-dUTP, was detectable in AD 198-treated 32D.3 and 32D.3/Bcl-2 cells within 30 min of drug exposure (Fig. 5). End labeling was observed in greater than 95% of 32D.3 and 32D.3/Bcl-2 cells 2 h after drug exposure. DNA fragmentation, after AD 288 treatment, was observed in 32D.3 cells 12 h after drug exposure but to a significant extent only after 48 h in 32D.3/Bcl-2 cells. More detailed analysis of DNA fragmentation by pulse-field gel electrophoresis correlated positive TUNEL staining in AD 198-treated cells with the generation of 50–300-kb DNA fragments, also indicative of an apoptotic cellular response (Ref. 45; Fig. 6). Generation of large DNA fragments was detectable within 1 h of AD 198 treatment of 32D.3 cells and intensified with increasing time. 32D.3/Bcl-2 cells exhibited comparable DNA fragmentation beginning at 2 h after drug exposure, and this intensified over time. AD 198-mediated cell death was also caspase dependent (see below).

These previous results led to the question of how AD 198 and AD 445 were able to circumvent the antiapoptotic effects of Bcl-2 and Bcl-X<sub>L</sub>. This could be attributed to reduced Bcl-2 activity through down-regulation or to modulation of posttranslational modification, circumvention of mitochondrial involvement in apoptosis, or activation of mitochondrial-dependent apoptosis despite the presence of Bcl-2. Throughout the time course of apoptosis, the overall amount of Bcl-2 remained unchanged, as did the electrophoretic mobility (Fig. 7), which suggested that Bcl-2 was neither cleaved by caspase-3, nor hyperphosphorylated and inactivated, as observed in response to Taxol (14). Incubation of 32D.3/Bcl-2 cells with <sup>32</sup>P<sub>i</sub> failed to radioactively label Bcl-2 in either treated or untreated cells (data not shown). The results suggest that AD 198 induces apoptosis in the presence of intact and, presumably, active Bcl-2. We next assessed the extent of mitochondrial involvement in apoptosis induced by AD 198 by monitoring the release of cytochrome c into the cytosol, and by assessing the downstream activation of caspase-3 and resulting cleavage of PARP (Fig. 8). Cytochrome c was observed in the cytosol of both 32D.3 and 32D.3/Bcl-2 cells within 1 h after AD 198 treatment. Release of a M<sub>r</sub> 17,000 active fragment after caspase-3 cleavage (46)

was, likewise, detected within 2 h after AD 198 treatment of both 32D.3 and 32D.3/Bcl-2 cells and was paralleled by caspase-mediated PARP cleavage to release a M<sub>r</sub> 85,000 fragment, again with a slight delay of 1 h observed in 32D.3/Bcl-2 cells (Fig. 8). The results indicate that AD 198 induces mitochondrial-mediated apoptosis despite the presence of functional Bcl-2.

Given the ability of AD 198 to induce rapid PKC- $\alpha$  and - $\delta$  translocation in NIH3T3 cells (23), the correlation of 14-O-acyl chain length with PKC binding and modulation of activity (24), and the ability of AD 198 and AD 445 to circumvent Bcl-2, the role of PKC activity in AD 198- and AD 445-induced apoptosis was investigated more precisely. It has been previously shown that 32D.3 cells predominantly express PKC- $\alpha$ , - $\delta$ , and - $\eta$ , with very low levels of PKC- $\beta$  and - $\zeta$ . PKC- $\alpha$  and high levels of PKC- $\delta$  appear to be involved in phorbol ester-induced myeloid differentiation and cross-regulation of PKC expression in 32D.3 cells (47, 48). Furthermore, PKC- $\delta$  activation, in general, is involved in the induction of apoptosis (49). Therefore, we determined whether PKC- $\alpha$  and - $\delta$  were activated by AD 198, using PKC- $\alpha$  and - $\delta$  translocation to cell membrane and PKC- $\delta$  cleavage to its M<sub>r</sub> 40,000 catalytic fragment as indicators of enzyme activation (49). Within 30 min of AD 198 exposure, a modest increase in membrane-associated PKC- $\delta$  and - $\alpha$  was observed in both 32D.3 and 32D.3/Bcl-2 cells (Fig. 9A). Analysis of total cellular PKC- $\delta$  revealed that within 1 h of drug exposure and initial translocation, PKC- $\delta$  was cleaved to yield a M<sub>r</sub> 40,000 fragment (Fig. 9B). Translocation of PKC- $\delta$  to mitochondria, either as holoenzyme or cleaved catalytic fragment, has been reported to lead to depolarization and to the release of cytochrome c (32, 33, 35), whereas association of the M<sub>r</sub> 40,000 fragment with the nucleus leads to nuclear apoptotic changes (50). Therefore, we next determined whether AD 198 rapidly induced PKC- $\delta$  translocation to mitochondria and nuclei along with cleavage and activation of PKC- $\delta$  (Fig. 9C). Before drug treatment, mitochondria and nuclei of 32D.3 cells contained high levels of PKC- $\delta$  holoenzyme. Within 1 h of AD 198 exposure, no increase in total nuclear or mitochondrial PKC- $\delta$  was observed. However, PKC- $\delta$  cleavage was detectable with 1 h of exposure in both 32D.3 and 32D.3/Bcl-2 cells. Within 6 h after AD 198 exposure, mitochondrial and nuclear PKC- $\delta$  was extensively cleaved to the M<sub>r</sub> 40,000 catalytic fragment with a concomitant loss of

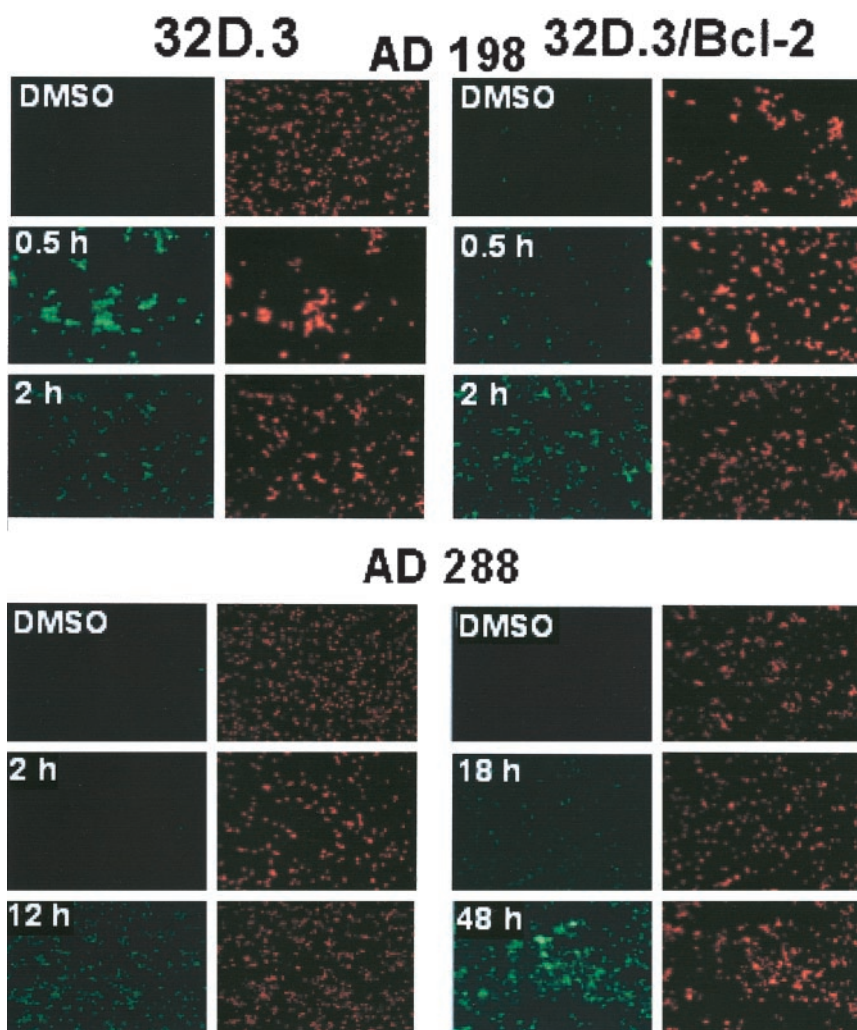
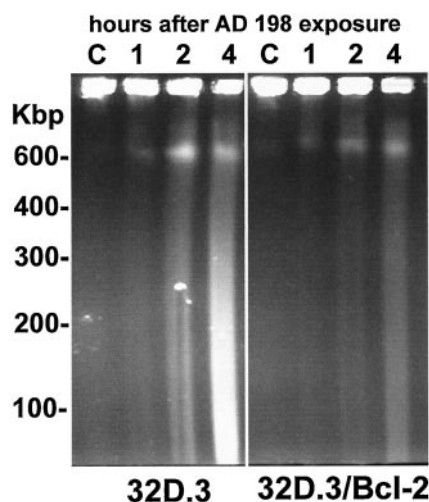


Fig. 5. Detection of DNA fragmentation in AD 198- and AD 288-treated 32D.3 cells by TUNEL assay. Cells were treated with  $5 \mu\text{M}$  drug or 1% DMSO alone (control) for 1 h, washed twice in warm PBS, incubated in drug-free medium for the time periods indicated, then prepared for 3'-biotinylation of fragmented DNA using the TUNEL assay procedure. Individual fields of cells were detected for both DNA 3'-end labeling (green) or total cellular DNA (red). Composite image is representative of three independent experiments.

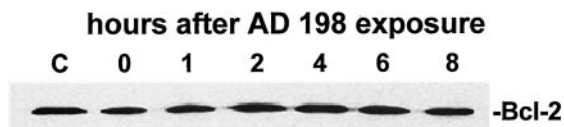
holoenzyme. Overall, these data suggested that AD 198 rapidly induces PKC- $\delta$  cleavage and activation, resulting in rapid mitochondrial recruitment, which triggers apoptotic events downstream of mitochondria. Because increased caspase activity is associated with PKC- $\delta$  cleavage and activation (35), we examined whether AD 198-induced apoptosis is dependent on both PKC- $\delta$  and caspase activities. Anthracycline treatment of 32D.3 and 32D.3/Bcl-2 cells, as described in Fig. 2A, in combination with the PKC inhibitor, rottlerin, at a concentration that is selective for PKC- $\delta$ , (51) resulted in 4- to 7-fold delays in 50% cell kill of AD 198 and AD 445 cytotoxicity in 32D.3 and 32D.3/Bcl-2, respectively (Fig. 10A). No comparable delay was achieved in DOX- or AD 288-treated cells. In contrast, treatment of 32D.3 and 32D.3/Bcl-2 cells with the PKC- $\alpha$  and - $\beta$  inhibitor, Gö 6976, did not delay DOX or AD 288 cytotoxicity, whereas AD 198 cytotoxicity was slightly enhanced (Fig. 10B). Similar results were observed with GF, a nonselective PKC inhibitor, although a slight but reproducible delay in DOX cytotoxicity was observed in both 32D.3 and 32D.3/Bcl-2 cells (Fig. 10C). Whereas GF did not delay cytotoxicity after  $5 \mu\text{M}$  AD 198 treatment, parallel studies using  $2.5 \mu\text{M}$  AD 198 in combina-

tion with GF showed a 10% delay in cytotoxicity (not shown). Similarly, GF and Gö 6976 also delayed the rate of 50% cell kill by AD 445 2-fold in 32D.3 cells and 4-fold in 32D.3/Bcl-2 cells. Cells treated with  $1 \mu\text{M}$  rottlerin, alone, were 70% viable after 72 h, whereas  $2 \mu\text{M}$  GF, alone, was noncytotoxic. Gö 6976 treatment, alone, resulted in 50% viability after 30 h for 32D.3 and in 70% viability after 72 h for 32D.3/Bcl-2. Consistent with the delay in cytotoxicity, PARP and PKC- $\delta$  cleavage were also effectively delayed from 1 to 20 h for both 32D.3 and 32D.3/Bcl-2 cells when treated with rottlerin (Fig. 11A). These results suggest that PKC- $\delta$  activation plays an important role in AD 198-induced apoptosis.

The cleavage of PKC- $\delta$  holoenzyme to yield the  $M_r$  40,000 catalytic fragment is caspase mediated in cells treated with UV radiation (52) or chemotherapeutic agents (53). To confirm that the cleavage of PKC- $\delta$  in AD 198-treated cells was likewise caspase mediated, 32D.3 and 32D.3/Bcl-2 cells were treated with the pan-caspase inhibitor, Z-VAD-fmk, before AD 198 treatment (Fig. 11B). Whereas extensive cleavage of PKC- $\delta$  was detected 3 h after AD 198 exposure in the absence of Z-VAD-fmk, pretreatment with Z-VAD-fmk completely inhibited PKC- $\delta$  cleavage. As expected, PARP



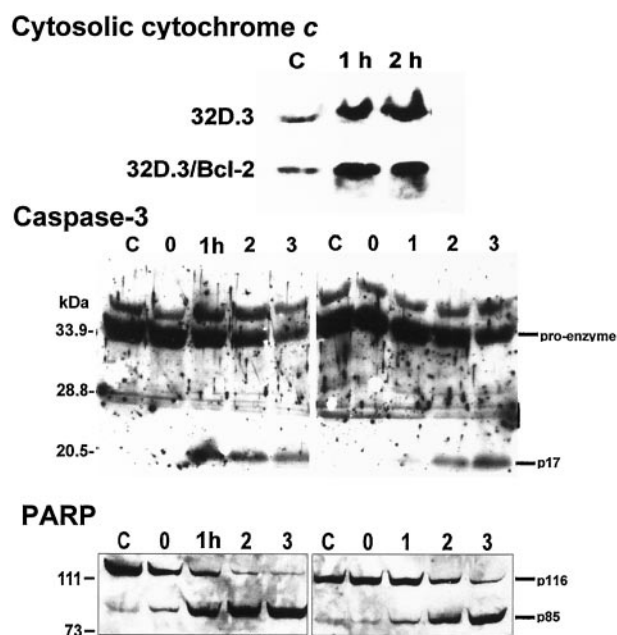
**Fig. 6.** Detection of high-molecular-weight DNA fragments in AD 198-treated 32D.3 cells by pulsed-field gel electrophoresis. 32D.3 and 32D.3/Bcl-2 cells were exposed to 5  $\mu$ M AD 198 for 1 h, followed by incubation in drug-free medium for 1, 2, or 4 h. Control (C) cells were exposed to 1% DMSO for 1 h, followed by incubation in drug-free media for 4 h. Cellular DNA was prepared for pulse-field gel electrophoresis. DNA was stained with ethidium bromide.



**Fig. 7.** Bcl-2 expression is not affected by AD 198 in 32D.3/Bcl-2 cells. 32D.3/Bcl-2 cells were treated with 5  $\mu$ M AD 198 for 1 h and then incubated in drug-free medium. At times indicated (*hours after AD 198 exposure*), 25- $\mu$ g samples of cell lysates were subjected to electrophoresis through 10% polyacrylamide, blotted onto nitrocellulose, and probed with antihuman Bcl-2 antibody as described in Fig. 2. C, control.

cleavage was also delayed in AD 198-treated 32D.3 and 32D.3/Bcl-2 cells pretreated with Z-VAD-fmk. Pretreatment of 32D.3 and 32D.3/Bcl-2 cells with Z-VAD-fmk, followed by a continuous exposure to AD 198 for 6 h, also resulted in a 2- and 1.5-fold delay in cytotoxicity for 32D.3 and 32D.3/Bcl-2 (data not shown).

In an effort to determine at which point in the AD 198-mediated apoptotic signaling pathway rottlerin and Z-VAD-fmk were inhibitory, we determined whether mitochondrial membrane depolarization by AD 198 required the caspase-mediated cleavage of PKC- $\delta$ , or whether active holoenzyme was sufficient (Fig. 12). In viable cells, DePsiher penetrates polarized mitochondrial membranes and produces a red punctate pattern, as detected by fluorescence microscopy (No Drug). After 1 h of AD 198 treatment, 32D.3 cells did not exhibit punctate fluorescence, but merely diffuse cytoplasmic fluorescence, produced by cytoplasmic AD 198 distribution. The cell shrinkage characteristic of apoptotic cells is notable. Pretreatment of 32D.3 cells with Z-VAD-fmk before AD 198, as described in Fig. 11, did not inhibit mitochondrial depolarization, as demonstrated by the loss of mitochondrial staining by DePsiher. However, pretreatment of 32D.3 cells

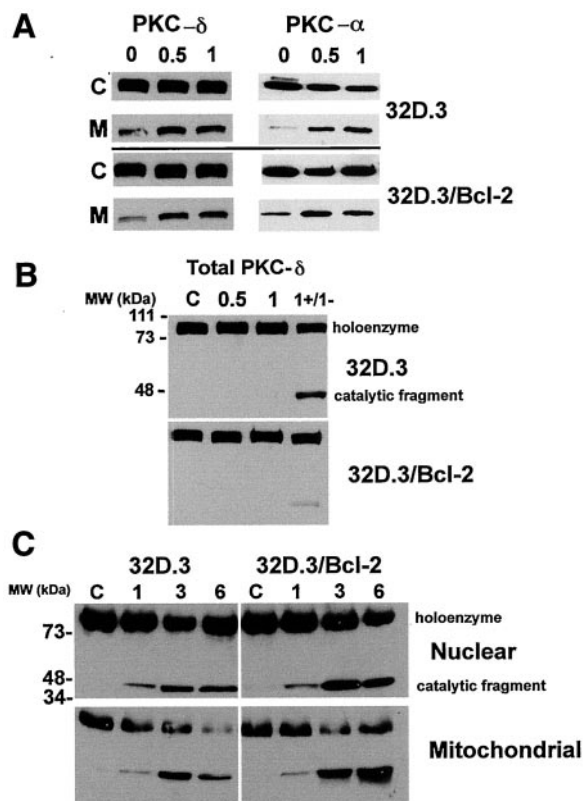


**Fig. 8.** Immunoblot analysis of cytochrome c, caspase 3, and PARP in AD 198-treated 32D.3 and 32D.3/Bcl-2 cells. Cells were exposed to 5  $\mu$ M AD 198 or 1% DMSO for 1 h, followed by incubation in drug-free medium. At the times indicated, cells were fractionated, subjected to SDS-PAGE, and transferred to nitrocellulose. Filters were treated with either anti-cytochrome c monoclonal antibody (1:250; PharMingen, San Diego, CA) for 2 h, anti-caspase-3 polyclonal antibody (1:200; Santa Cruz Biotechnology) for 1.5 h, or anti-PARP monoclonal antibody (1:100; Oncogene Research Products, Cambridge, MA) for 1 h, followed by 1-h treatment with 1:1000 dilution of goat anti-mouse-HRP-conjugated second antibody or 1:3000 dilution of goat antirabbit-HRP conjugated second antibody and lumigen. Reactive bands were observed by exposure of filters to X-ray film. Composite image is representative of three independent experiments.

with rottlerin, as described in Fig. 10, resulted in the persistence of punctate fluorescence, indicating preservation of mitochondrial membrane potential. These results suggest that PKC- $\delta$  holoenzyme activation, and not cleavage, is required for AD 198-mediated mitochondrial involvement in apoptotic signaling.

## Discussion

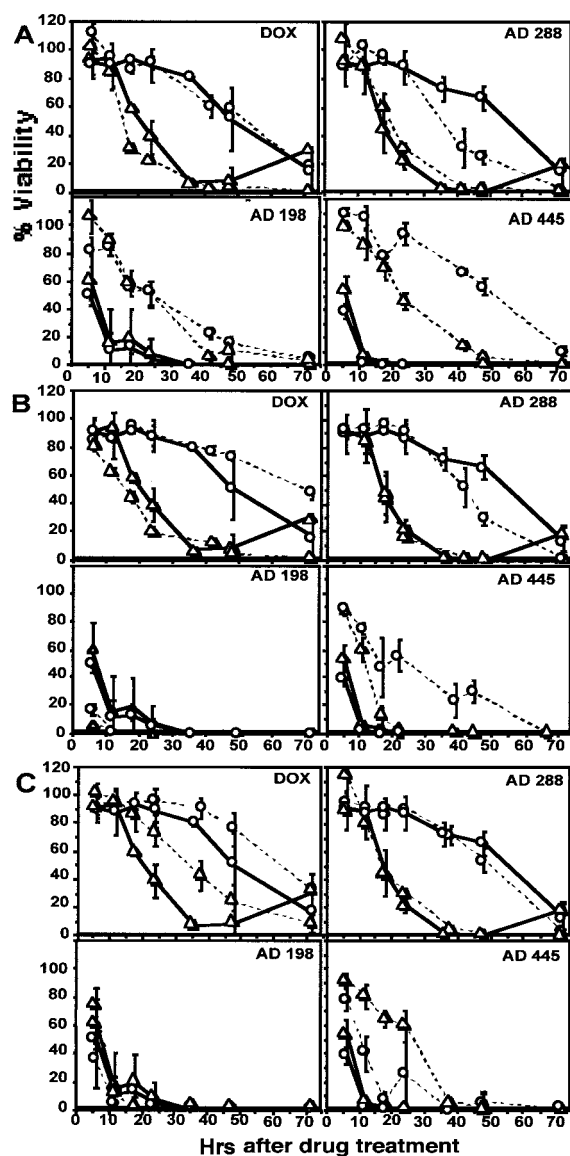
Suppression of apoptotic signaling by Bcl-2 expression is a serious impediment to chemotherapeutic eradication of cancer cells. Current clinical anticancer agents typically damage DNA, membrane, or cytoskeletal components and, subsequently, rely on that damage to trigger growth arrest and apoptosis. The inability of chemotherapeutic agents, such as DOX, to induce apoptosis may arise from either insufficient cellular damage to activate apoptosis in favor of arrest and repair through transport-mediated resistance, a functional defect in one of several important members of this apoptotic cascade, such as p53 protein or cyclin kinase inhibitors, or the inappropriate expression of proteins that block the apoptotic signal cascade, such as Bcl-2 (8). We have previously demonstrated that, unlike DOX or daunorubicin, AD 198 and AD 445 are not transported by either P-glycoprotein or multidrug resistance protein (MRP), which is attributable, at least



**Fig. 9.** Translocation of PKC- $\delta$  and - $\alpha$  in AD 198-treated 32D.3 cells. **A**, 32D.3 and 32D.3/Bcl-2 cells were treated with 5  $\mu$ M AD 198 for 0.5 or 1 h and fractionated to enrich for cytosolic (C) or membrane (M) components. Protein was subjected to SDS-PAGE, transferred to nitrocellulose, and probed with either a 1:1000 dilution of anti-PKC- $\delta$  polyclonal antibody for 1 h, or a 1:1000 dilution of anti-PKC- $\alpha$  monoclonal antibody for 2 h (Upstate Biotechnology). Chemiluminescent detection was carried out as described in Fig. 2. **B**, cells were treated with 5  $\mu$ M AD 198 for 0.5 h, 1 h, and 1 h followed by 1 h off the drug (1+/1-). Total cell lysates were prepared for PKC- $\delta$  immunoblot analysis as described for A. **C**, 32D.3 and 32D.3/Bcl-2 cells were treated with 5  $\mu$ M AD 198 for 1 h, followed by incubation in drug-free medium. At the times indicated, cells were fractionated to enrich for mitochondrial and nuclear components. Samples were prepared for PKC- $\delta$  immunoblot analysis as described for A. Composite image is representative of at least three independent experiments. MW (kDa),  $M_r$  in thousands.

in part, to their highly lipophilic structure (26, 28). Furthermore, because AD 198 and AD 445 do not target topo II, cells expressing the altered topoisomerase multidrug resistance phenotype remain sensitive to these agents (29). In this report, we have demonstrated that the rate at which the acylantracycline analogues AD 198 and AD 445 induce rapid mitochondrial- and caspase-dependent apoptosis, and the concentration required to achieve 90% cell kill are unaffected by the overexpression of Bcl-2 or Bcl-X<sub>L</sub>. These findings thus identify an additional potential therapeutic benefit of AD 198 and AD 445 in the circumvention of cellular drug resistance.

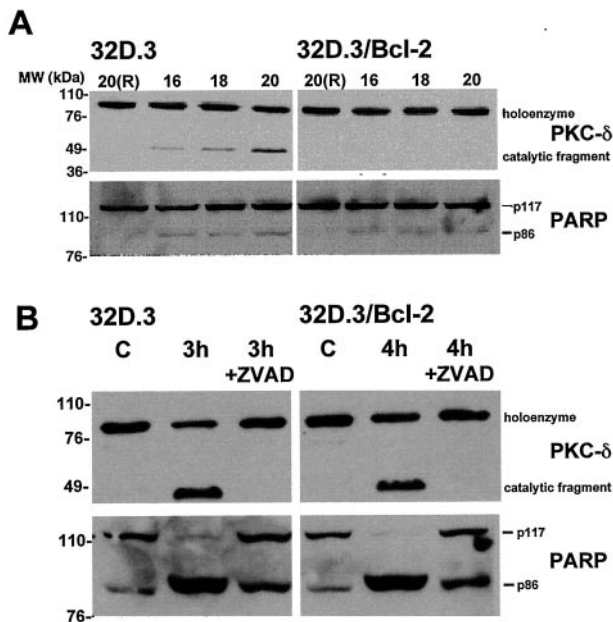
Antiapoptotic Bcl-2 proteins are reported to block apoptosis through multiple mechanisms, including interactions with pro-apoptotic Bcl-2 family proteins, inhibiting cytochrome c and apoptosis-inducing-factor release, and regulating the binding of Apaf-1 to the mitochondrial outer mem-



**Fig. 10.** Effect of PKC inhibitors on anthracycline cytotoxicity in 32D.3 cells. Cells were pretreated for 2 h with either 10  $\mu$ M rottlerin (A), 1  $\mu$ M Gö 6976 (B), or 2  $\mu$ M GF (C), followed by 1-h 5- $\mu$ M anthracycline exposure and incubation in anthracycline-free medium in the presence of PKC inhibitors. Viability was determined by trypan blue staining as described in Fig. 2. Each datum point, the mean ( $\pm$ SE) of at least three independent determinations, each consisting of 300–500 cells/count. — $\Delta$ —, 32D.3 cells; — $\circ$ —, 32D.3/Bcl-2 cells; — $\square$ —, 32D.3 cells + PKC inhibitor; — $\circ$ —, 32D.3/Bcl-2 cells + PKC inhibitor.

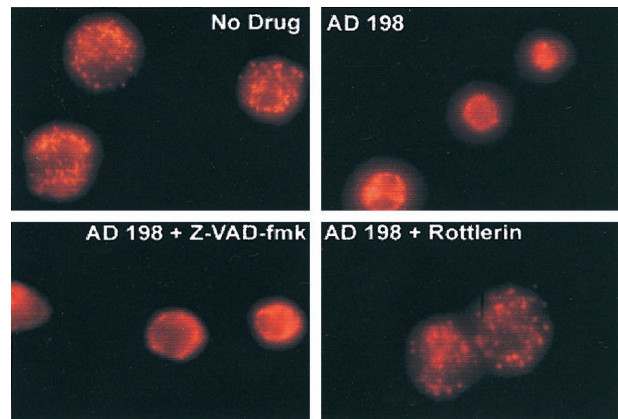
brane surface (5). The circumvention of Bcl-2 activity can be achieved through one of several pathways, including the inactivation of Bcl-2, the induction of mitochondrial-independent apoptosis, the induction of mitochondrial-dependent apoptosis despite the presence of Bcl-2, or by necrosis. Bcl-2 activity can be regulated at both the transcriptional and posttranslational levels. Phorbol ester treatment under conditions that decrease PKC activity and the PKC inhibitor H-7 reportedly down-regulate the expression of Bcl-2 mRNA and protein in myeloid cells (54, 55). The effect of AD 198 and AD





**Fig. 11.** Effect of the PKC- $\delta$  inhibitor rottlerin and the pan-caspase inhibitor Z-VAD-fmk on PKC- $\delta$  and PARP cleavage. **A**, cells were preincubated with 10  $\mu$ M rottlerin for 2 h, followed by a 1-h exposure to 5  $\mu$ M AD 198 or an equal volume of DMSO for control cells. Cells were washed twice in warm PBS, then incubated in medium containing 10  $\mu$ M rottlerin for 16, 18, or 20 h after AD 198 exposure; control cells were incubated for 20 h in 10  $\mu$ M rottlerin. Immunoblot analyses of PKC- $\delta$  and PARP were performed as described in Fig. 8. 20(R), 20-h treatment with rottlerin alone. Cells were preincubated with 200  $\mu$ M Z-VAD-fmk for 1 h, followed by continuous exposure to 5  $\mu$ M AD 198 or an equal volume of DMSO for control cells. **B**, cells were washed twice in warm PBS, then incubated in medium containing 200  $\mu$ M Z-VAD-fmk for 3 h or 4 h after AD 198 exposure. Samples 3 h and 4 h were treated with 5  $\mu$ M AD 198 as above, but without Z-VAD-fmk treatment. Immunoblot analyses of PKC- $\delta$  and PARP were performed as described in Fig. 8. Composite image is representative of at least three independent experiments. MW (kDa),  $M_r$  in thousands.

445 on endogenous Bcl-2 gene expression has not yet been determined. However, as we have demonstrated, the circumvention of Bcl-2-mediated inhibition of apoptosis by AD 198 and AD 445 does not require suppression of Bcl-2 expression, because protein levels remain unchanged after drug treatment. In addition, Bcl-2 remains intact after drug treatment, which indicates that Bcl-2 antiapoptotic function is not inhibited by caspase-3-mediated cleavage of Bcl-2 to produce a pro-apoptotic fragment (56). Taxol has been shown to induce the phosphorylation of Bcl-2 at multiple serine and threonine residues, resulting in reduced heterodimerization with Bax and decreased antiapoptotic activity (14, 57). Phosphorylation of Bcl-2 has been attributed to multiple kinases, including PKC (17), and is detectable by decreased electrophoretic mobility as phosphate groups are added (14, 16, 57). However, the absence of multiple electrophoretic species after AD 198 treatment and the inability to isotopically label Bcl-2 with [ $^{32}$ P]-P $_i$  suggests that AD 198 and AD 445 do not inactivate Bcl-2 through phosphorylation. The rapid induction of apoptosis by AD 198 in the presence of Bcl-2 alternatively suggests that AD 198 may trigger apoptosis through Fas-mediated activation of down-



**Fig. 12.** Effect of the PKC- $\delta$  inhibitor rottlerin and the pan-caspase inhibitor Z-VAD-fmk on mitochondrial membrane depolarization. 32D.3 cells were untreated (*No drug*), treated with 5  $\mu$ M AD 198 for 1 h (*AD 198*), or treated with 5  $\mu$ M AD 198 and either ZVAD-fmk or rottlerin as described in Fig. 11. All of the cells were then washed in warm PBS and then incubated in AD 198-free medium for 2 h. Cells were then stained with DePsipher for 20 min and observed by fluorescence microscopy. Composite image is representative of at least three independent experiments.

stream signaling that is mitochondrial independent and not under Bcl-2 control (58). The Fas pathway requires the FADD linker protein to allow binding of procaspase-8 to the death-inducing signaling complex (DISC) for subsequent activation (59). Whereas myeloid cells that are treated with DOX do not appear to require Fas activation for apoptotic signaling (60, 61), we considered the possibility that the localization of AD 198 in the Golgi-rich perinuclear region of the cells induces the release of Fas ligand and the subsequent stimulation of Fas-mediated signaling (62). This scenario is, however, highly unlikely to occur, because 32D.3 cells are FADD deficient.<sup>4</sup> The rapid depolarization of mitochondrial membranes, the release of mitochondrial cytochrome c, and subsequent activation of caspase-3 also indicate that AD 198 triggers mitochondrial involvement in apoptotic signaling despite Bcl-2 expression. In light of these observations, it is likely that AD 198 and AD 445 simply overcome the antiapoptotic effects of Bcl-2 and induce rapid, mitochondrial-mediated apoptosis. The marginal, but reproducible 1 h delay in DNA fragmentation, caspase-3 cleavage, and PARP cleavage in Bcl-2-expressing 32D.3/Bcl-2 cells suggest that Bcl-2 may, somehow, briefly delay AD 198-mediated apoptosis.

We have not completely ruled out the inhibition of Bcl-2 by direct drug interaction, as has been reported for antimycin A and HA 14-1 (63, 64). Small molecules have been reported to inhibit Bcl-2 through direct binding. Modeling studies suggest that antimycin A binds to the BH3 domain of Bcl-X $_L$  to induce mitochondrial swelling and the loss of mitochondrial membrane potential (63). HA 14-1 is a small polycyclic aromatic compound capable of binding to a surface pocket of Bcl-2 and impairs its antiapoptotic functions (64). Minor similarities between the chemical structures of antimycin A, HA

<sup>4</sup> J. L. Cleveland, unpublished observations.

14-1, and AD 198 hint at the potential of AD 198 to directly bind to Bcl-2 and modulate its activity. However, our findings with the PKC- $\delta$  inhibitor, rottlerin, suggest that PKC- $\delta$ , rather than Bcl-2, is a principal target for AD 198 and AD 445.

The ability of AD 198 and AD 445 to both trigger rapid apoptosis and circumvent Bcl-2 correlates with the binding to, and activation of, PKC- $\delta$ . Previous studies have indicated the structural importance of the 14-*O*-valerate substitution in permitting AD 198 to bind to the C1 domain of PKC- $\delta$ , based on computer modeling (23) and cell-free competitive binding studies using [ $^3$ H]-PDBu (24). The carbonyl of the 14-*O*-valerate is involved in hydrogen bonding to the PKC- $\delta$  C1 domain, whereas the acyl chain appears to stabilize the membrane-bound protein-ligand complex in a manner analogous to the phorbol ester acyl moieties. Indeed, the diacylglycerol/phorbol ester pharmacophore has been identified within the combined valerate side chain and chromophore A ring of AD 198 (23). Consequently, decreasing 14-*O*-acyl chain length leads to protein-ligand destabilization, as evidenced by decreased competitive inhibition of phorbol ester binding to PKC- $\delta$ , and results in decreased modulation of PKC activity. The correlation of decreasing chain length with a reduced ability to circumvent Bcl-2 indicates that PKC binding by AD 198 and AD 445 is essential to overcome the antiapoptotic effects of Bcl-2. Rather than alter Bcl-2 activity, we suggest that AD 198 and AD 445 overwhelm Bcl-2 activity through potent PKC-mediated apoptotic signaling. This is supported by the observation that rottlerin inhibits AD 198 and AD 445 cell kill in both Bcl-2-expressing and control 32D.3 cells. Although the ability of rottlerin to selectively and directly inhibit PKC- $\delta$  activity has recently been questioned (65, 66), preliminary studies in our laboratory using a TDN PKC- $\delta$  vector (67) transfected into 32D.3 cells support our studies with rottlerin. TDN clones exhibiting a 43% decrease in PKC- $\delta$  activity relative to empty vector transfectants showed a delay in AD 198 (5  $\mu$ M/1 h) 50% cell kill from 8 h in empty vector control cells to 18 h in TDN cells.<sup>5</sup> Therefore, PKC- $\delta$  appears to be the principal cytotoxic target of AD 198 and AD 445 in 32D.3 cells. PKC- $\delta$  expression is abundant in 32D.3 cells as well as most hematopoietic cells. PKC- $\alpha$  is also highly expressed, whereas only small amounts of PKC- $\eta$  and no PKC- $\beta$  or - $\epsilon$  are detectable (47, 68). The apoptotic activity of PKC- $\delta$  as a holoenzyme and as a cleaved  $M_r$  40,000 catalytic fragment has been well documented in a variety of cell lines (30, 32, 33). In 32D.3 cells, overexpression of PKC- $\delta$  and subsequent activation by phorbol ester results in myeloid differentiation to macrophages rather than apoptosis (47). This specific effect has been attributed to the phosphorylative inhibition of JAK2 tyrosine kinase by the catalytic domain of PKC- $\delta$  (68, 69). Whereas AD 198 and phorbol esters both bind to the C1 domain of PKC- $\delta$ , the resulting cellular effects are clearly different. 12-*O*-tetradecanoylphorbol-13-acetate treatment of 32D.3 cells for 50 h activates PKC- $\delta$  without evidence of proteolytic cleavage

(68), whereas AD 198 and AD 445 both induce cleavage to produce the  $M_r$  40,000 catalytic fragment within 1 h of treatment. One interpretation of these results is that activation of PKC- $\delta$  by AD 198 and AD 445 may be through potentiation of proteolytic cleavage rather than through activation of holoenzyme. The binding of phorbol esters to the C1 domain of PKC not only activates but alters protein conformation to expose the V3 region, facilitating proteolytic cleavage by calpain I to release the catalytic subunit (70). To that end, AD 198 and AD 445 may make PKC- $\delta$  more susceptible to caspase-3 cleavage in the V3 region (53), resulting in accelerated PKC- $\delta$ -mediated apoptotic signaling. Consistent with this notion is the inhibition of AD 198-mediated PKC- $\delta$  cleavage by the pan-caspase inhibitor, Z-VAD-fmk. However, the ability of Z-VAD-fmk to effectively block PKC- $\delta$  cleavage after AD 198 treatment, although unable to block mitochondrial depolarization, indicates that PKC- $\delta$  cleavage is not required for AD 198 recruitment of the mitochondria into the apoptotic pathway. Rottlerin inhibition of depolarization, however, suggests that PKC- $\delta$  holoenzyme activation is the initial apoptotic step and that PKC- $\delta$  cleavage may be a secondary feedback effect. Whereas it has been previously reported that the pro-apoptotic effects of PKC- $\delta$  are blocked by Bcl-2 and Bcl-X<sub>L</sub> (71), no such block exists after AD 198 or AD 445 treatment, which suggests that the extent of PKC- $\delta$  activation exceeds the antiapoptotic potency of Bcl-2 or that PKC- $\delta$  induces mitochondrial depolarization and cytochrome c release after AD 198 or AD 445 treatment in a manner that is not inhibited by Bcl-2.

The lack of a comparable delay in DOX or AD 288 cytotoxicity by rottlerin treatment, despite the eventual cleavage and activation of PKC- $\delta$ , indicates that PKC- $\delta$  activity is critical only for AD 198 and AD 445 induction of apoptosis. Rottlerin pretreatment of AD 198-treated cells blocks both PKC- $\delta$  and PARP cleavage, indicating that PKC- $\delta$  cleavage and activation may be essential to highly activate caspase-3. In a similar fashion, others have reported that rottlerin treatment of C5 salivary acinar cells blocked both PKC- $\delta$  cleavage and apoptosis induced by etoposide (35).

AD 198 and AD 445 do not interact exclusively with PKC- $\delta$  but are likely to bind to other conventional and novel PKC isozymes. Indeed, studies in our laboratories examining the effect of AD 198 on LLC-PK renal epithelial cells have shown that AD 198, but not AD 288, cytotoxicity increases coordinately with tetracycline-controlled expression of PKC- $\alpha$ . Furthermore, we have demonstrated that both Gö 6976 and GF modulate AD 445 cytotoxicity, whereas GF delay of cytotoxicity occurs at lower concentrations of AD 198. It is not yet clear how this occurs. Gö 6976 and GF are selective for PKC- $\alpha$  and - $\beta$ , with inhibition of PKC- $\delta$  by GF at concentrations 30-fold higher than for PKC- $\alpha$ . The delay of cytotoxicity by Gö 6976 and GF would suggest that AD 445 and AD 198 also modulate PKC- $\alpha$  activity in 32D.3 cells in a manner that contributes to drug cytotoxicity. The structural differences between AD 198 and AD 445 may confer differing preferences for PKC isozymes, resulting in differential effects. In support of this concept, initial translocation studies performed in

<sup>5</sup> C. M. Barrett, T. W. Sweatman, and L. Lothstein. Specific inhibition of protein kinase C- $\delta$  holoenzyme activity in the mitochondria of murine myeloid cells delay apoptosis induced by *N*-benzyladriamycin-14-valerate (AD 198), manuscript in preparation.

32D.3 cells with AD 445 reveal the translocation of PKC- $\alpha$  to the detergent-insoluble fraction within 1 h after drug exposure, whereas little, if any, commensurate translocation was observed with AD 198, AD 288, or DOX.<sup>6</sup>

Whereas PKC proteins are clearly involved in AD 198-induced apoptosis, the possibility that other proteins, containing C1 domains, play a role cannot be discounted. AD 198 has also been shown to competitively inhibit phorbol ester binding to  $\beta$ 2-chimaerin (24), a non-kinase phorbol ester receptor containing a C1 domain. Theoretically, AD 198 can bind to any protein containing a C1 domain such as other  $\beta$ -chimaerins (Rac-GTPase-activating proteins), ras guanyl-releasing protein (Ras-GRP), Unc-13/Munc-13 protein family involved in exocytosis, and protein kinase D (72, 73). However, with the use of PKC inhibitors, we have shown that PKC- $\delta$  and, to a lesser extent, PKC- $\alpha$  are principal signaling components of AD 198/AD 445-induced apoptosis in 32D.3 cells. The PKC inhibitors bind to the regulatory region of the protein; thus they have no effect on proteins that only contain a C1 domain, thereby reducing the potential role that these proteins play in the mechanism of apoptosis.

These AD 198 and AD 445 findings represent a significant step in the development of potential chemotherapeutic agents, the cytotoxicity of which is unaffected by Bcl-2 expression. Few other compounds presently exist. Of interest is the synthetic flavone, flavopiridol, which demonstrates circumvention of Bcl-2 in transfected HeLa cells after an 18-h drug exposure (9). Flavopiridol appears to require caspase-8 activity to induce apoptosis. However, given the absence of FADD in 32D.3 cells, we believe it is unlikely that caspase-8 activation is essential for AD 198-induced apoptosis.

In summary, AD 198 and AD 445 rapidly trigger apoptosis in 32D.3 myeloid cells by inducing the activation of PKC- $\delta$ , which results in mitochondrial involvement in the apoptotic signaling cascade. The speed and potency of drug-mediated signaling allows the circumvention of Bcl-2 and Bcl-X<sub>L</sub> anti-apoptotic activity to induce cytochrome *c* release. The rapidity with which apoptosis occurs and the inability of loss of p53 function to impede AD 198 cytotoxicity<sup>6</sup> suggests that AD 198 and AD 445 trigger apoptosis not by inflicting cellular damage, but by directly and potentially activating the apoptotic signaling cascade.

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