N-benzyladriamycin-14-valerate (AD 198) activates protein kinase C-δ holoenzyme to trigger mitochondrial depolarization and cytochrome *c* release independently of permeability transition pore opening and Ca^{2+} influx

Leonard Lothstein, Luydmila Savranskaya, Christina M. Barrett*, Mervyn Israel and Trevor W. Sweatman

Unlike nuclear-targeted anthracyclines, the extranuclear-targeted doxorubicin congener, *N*-benzyladriamycin-14-valerate (AD 198), does not interfere with normal topoisomerase II activity, but binds to the C1b regulatory domain of conventional and novel isoforms of protein kinase C (PKC). The resulting interaction leads to enzyme activation and rapid apoptosis in a variety of mammalian cell lines through a pathway involving mitochondrial events such as membrane depolarization ($\Delta\psi_m$) and cytochrome *c* release. Unlike other triggers of apoptosis, AD 198-mediated apoptosis is unimpeded by the expression of Bcl-2 and Bcl-X_L. We have further examined AD 198-induced apoptosis in 32D.3 mouse myeloid cells to determine how the anti-apoptotic effects of Bcl-2 are circumvented. The PKC-δ inhibitor, rottlerin, and transfection with a transdominant-negative PKC-δ expression vector both inhibit AD 198 cytotoxicity through inhibition of $\Delta\psi_m$ and cytochrome *c* release. While the pan-caspase inhibitor Z-VAD-FMK blocks AD 198-induced PKC-δ cleavage, however, it does not inhibit $\Delta\psi_m$ and cytochrome *c* release, indicating that AD 198 induces PKC-δ holoenzyme activation to achieve apoptotic mitochondrial effects. AD 198-mediated $\Delta\psi_m$ and cytochrome *c* release are also unaffected by cellular treatment with either the mitochondrial permeability transition pore complex (PTPC) inhibitor cyclosporin A or the Ca^{2+} chelators EGTA and BAPTA-AM. These results suggest that AD 198 activates PKC-δ holoenzyme, resulting in $\Delta\psi_m$ and cytochrome *c* release through a mechanism that is independent of both PTPC activation and Ca^{2+} flux across the mitochondria. PTPC-independent mitochondrial activation by AD 198 is consistent with the inability of Bcl-2 and Bcl-X_L expression to block AD 198-induced apoptosis. *Anti-Cancer Drugs* 17:495–502

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Department of Pharmacology and The UT Cancer Institute, University of Tennessee Health Science Center, Memphis, Tennessee, USA.

Correspondence to L. Lothstein, Department of Pharmacology, University of Tennessee Health Science Center, 874 Union Avenue, Memphis, TN 38163, USA.
Tel: +1 901 448-3334; fax: +1 901 448-7206;
e-mail: llothstein@utmem.edu

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Introduction

Changes in mitochondrial structure and function play a critical role in the potentiation of apoptotic signaling initiated by multiple stimuli [1]. Interaction of mitochondria with either pro-apoptotic members of the Bcl-2 family of proteins [2], caspases [3] or cytotoxic compounds directly, such as betulinic acid [4], can induce the formation of large multi-component pore complexes [permeability transition pore complex (PTPC)] whose assembly and activity accompany the loss of mitochondrial membrane potential ($\Delta\psi_m$), and the release of cytochrome *c*, apoptosis-inducing factor (AIF), Smac/DIABLO and Ca^{2+} from within the mitochondria. Cytochrome *c* and AIF, in turn, facilitate the proteolytic activation of caspases-6, -9 and -3, and nuclear endonucleases [5], while Smac/DIABLO facilitates caspase activation by inhibiting inhibitors of apoptosis proteins [6]. The PTPC is a multimeric protein complex formed by the association of the voltage-dependent anion channel (VDAC) in the outer membrane with the adenine nucleotide translocator (ANT) in the inner membrane along with other mitochondrial proteins including cyclophilin D (CyP-D), benzodiazepine receptor and hexokinase [1]. Expression of the pro-apoptotic members of the Bcl-2 family of proteins (e.g. Bax, Bad, Bid, Bak and Bcl-X_S), alone, is sufficient to promote cytochrome *c* release and apoptosis [7]. Bax, for example, is reported to insert into the outer mitochondrial membrane, and promote PTPC formation and opening, leading to $\Delta\psi_m$ [8]. Critical to these mitochondrial apoptotic events is the flux of Ca^{2+} across mitochondrial membranes. Ca^{2+} uptake from the extracellular stores

*Present address: GTx Inc., Memphis, Tennessee, USA.

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and released endoplasmic reticulum (ER) stores can lead to Ca^{2+} overload within mitochondria and induce the association of CyP-D with ANT, resulting in PTPC opening [9]. The influx of Ca^{2+} may proceed through several routes: influx through the PTPC itself [9], through Ca^{2+} uniporters in the inner membrane [10], through non-selective cation channels [11] or through a mechanism termed rapid mode of Ca^{2+} uptake, so far identified in hepatocytes and cardiomyocytes [12]. Consequently, pro-apoptotic mitochondrial events are inhibited by: expression of anti-apoptotic Bcl-2 proteins that either close the VDAC protein of the PTPC, heterodimerize and inhibit the actions of Bax and other pro-apoptotic Bcl-2 proteins or block Ca^{2+} release from the ER [2]; cyclosporin A (CsA) and bongkrekic acid, which bind CyP-D in the PTPC to block opening [1]; or Ca^{2+} chelators that prevent mitochondrial Ca^{2+} influx [11]. Thus, elevated expression of anti-apoptotic Bcl-2 proteins or pre-treatment of cells with these agents will block the mitochondrial-mediated activation of caspases required for the effector stage of apoptosis and preserve cell viability.

Mitochondrial involvement in apoptotic signaling is also mediated by members of the C family of Ser/Thr protein kinases (PKC) [13]. Pro-apoptotic activity is observed with PKC-α [14], PKC-δ [15–17] and PKC-ε [17]. In particular, PKC-δ activity is associated with apoptosis induced by chemotherapeutic agents, radiation, oxidative stress, death receptor ligands and phorbol esters [18]. The pro-apoptotic effects of PKC-δ are reported to be both a consequence of caspase-3-mediated cleavage of the catalytic domain from the regulatory regions [19], as well as the activation of PKC-δ holoenzyme [16]. Under both circumstances, PKC-δ activity coincides with the translocation of PKC-δ to the mitochondrial membrane [19–21]. The target molecules of activated PKC-δ are not fully identified, but are reported to include: c-Abl, resulting in $\Delta\psi_m$ and necrotic-like cell death [22]; p73β tumor-suppressor protein, phosphorylated by the caspase-generated catalytic fragment [23]; lamin, whose phosphorylation results in nuclear lamina disassembly during apoptosis [24]; the mitochondrial enzyme phospholipid scramblase 3 (PLS3), whose phosphorylation is associated with mitochondrial-dependent apoptosis [25]; and DNA-dependent protein kinase [26]. PKC-δ-mediated induction of apoptosis through mitochondrial association is blocked by the expression of anti-apoptotic Bcl-2 proteins [16] and renders those cells resistant to apoptosis triggered by various stimuli, including anti-tumor anthracyclines [27].

In previous studies we have shown that the novel, extranuclear-targeted anthracyclines *N*-benzyladriamycin-14-valerate (AD 198) and *N*-benzyladriamycin-14-pivalate (AD 445) trigger rapid apoptosis in a variety of cell lines in a manner that circumvents multiple mechanisms of drug resistance, including overexpression of the multidrug transporters P-glycoprotein and MRP [28–29], decreased topoisomerase II activity [30], p53 dysfunction [31], NF-κB activation [32], and the expression of Bcl-2 and Bcl-X_L [31–32]. Unlike nuclear-targeted anthracyclines such as doxorubicin (DOX) or epirubicin, AD 198 and AD 445 do not inflict DNA damage through inhibition of topoisomerase II activity [28,30]. Rather, AD 198 and AD 445 bind to the C1b regulatory domain of conventional and novel PKC isozymes in a manner that competitively inhibits phorbol ester binding, but nevertheless activates PKC [31,33,34]. In 32D.3 murine myeloid cells, AD 198 and AD 445 rapidly trigger mitochondrial membrane depolarization and cytochrome *c* release within 1 h of treatment. Expression of transfected human Bcl-2 or Bcl-X_L has no effect in inhibiting AD 198- and AD 445-induced apoptosis, but inhibits DOX-mediated apoptosis. However, the PKC-δ-selective inhibitor, rottlerin, specifically inhibits AD 198 and AD 445 apoptosis, but has no effect on the cytotoxicity of DOX or *N*-benzyladriamycin (AD 288) – a topoisomerase II catalytic inhibitor, and the principal cellular biotransformation product of AD 198 and AD 445 [35]. In an effort to determine how AD 198 circumvents the anti-apoptotic effects of Bcl-2, we examined the mechanism by which AD 198 triggers $\Delta\psi_m$ and cytochrome *c* release. We found that both events are triggered independently of PTPC activity and Ca^{2+} flux, but require the activation of PKC-δ holoenzyme rather than the cleaved catalytic subunit.

Materials and methods

Cell culture and reagents

Interleukin (IL)-3-dependent 32D.3 murine myeloid cell lines, transfected with either empty SFFV expression vector [36] or SFFV-Bcl-2 (32D.3/Bcl-2), (generous gift of Dr John Cleveland, St Jude Hospital, Memphis, Tennessee, USA) described previously [37], were maintained in RPMI 1640 medium with L-glutamine (Atlanta Biologicals, Norcross, Georgia, USA) supplemented with 10% FBS (Atlanta Biologicals) and 40 U/ml IL-3 (Sigma, St Louis, Missouri, USA) [38]. DOX was obtained from Sigma, while AD 288 and AD 198 hydrochloride salts were prepared in this laboratory according to previously described procedures [30]. Rottlerin, Z-VAD-FMK, CsA, betulinic acid and BAPTA-AM were obtained from Calbiochem (La Jolla, California, USA). All drugs were dissolved in DMSO. The final maximum DMSO concentration used in drug treatments (2% for 5 h) is not cytotoxic.

Mitochondrial membrane depolarization

32D.3 cells were treated continuously with either 10 μmol/l rottlerin or 200 μmol/l Z-VAD-FMK for 5 h, or in combination with AD 198 for 3 h following a 2-h incubation in inhibitors. Cells were then collected and

washed twice in warm PBS and prepared for staining with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanin iodide (DePsipher; Trevigen, Gaithersburg, Maryland, USA) according to the manufacturer's instructions. Cellular red fluorescence was observed microscopically (Olympus model BX60) using a filter above 620 nm.

Immunoblot analysis of cytochrome *c* release from mitochondria

For cytochrome *c* release into cytosol, cells were treated with drug as described above. After incubation in AD 198 for the times indicated, cells were washed in cold PBS, resuspended in mitochondrial isolation buffer (250 mmol/l sucrose, 20 mmol/l HEPES–KO_4, pH 7.4, 1.5 mmol/l $MgCl_2$, 10 mmol/l KCl and 1 mmol/l EDTA, with 1 mmol/l PMSF, 20 μmol/l leupeptin, 50 μg/ml pepstatin A, 50 μg/ml aprotonin and 2 mmol/l DTT) and incubated on ice for 20 min. Cells were lysed with a Dounce homogenizer and the lysate was subjected to centrifugation at 14 200 *g* for 15 min at 4°C. The resulting supernatant containing the cytosolic fraction was lyophilized and then suspended in RIPA buffer (50 mmol/l Tris–HCl, pH 8.0, 150 mmol/l NaCl, 0.1% SDS, 1% NP-40 and 0.5% sodium deoxycholate). Following protein determination of the samples, 100 μg of protein was resolved by SDS–PAGE in 15% polyacrylamide. Immunoblot analysis was performed as described previously [31] using an anti-cytochrome *c* antibody (BD Biosciences, San Diego, California, USA).

Cell viability determination

32D.3 cells, at a density of 5×10^5/ml, were exposed to the IC_{90} concentrations (drug concentration required to kill 90% of 32D.3 cells 72 h after a 1-h drug treatment; 5 μmol/l) of AD 198 and AD 288 in complete medium for 1 h at 37°C in a 5% CO_2 humidified environment. Control cells were treated with 1% DMSO for 1 h. Following drug treatment, cells were washed twice with PBS and incubated further in drug-free media. At the times indicated, 75-μl aliquots of cell suspension were combined with 25 μl Trypan blue (Life Technologies, Rockville, Maryland, USA) and assayed microscopically for cellular dye exclusion.

Transdominant-negative PKC-δ vector transfection and kinase activity assay

32D.3 cells were stably transfected by electroporation with either non-recombinant pCEV expression vector or PKC-δK376R transdominant-negative PKC-δ [39], both a kind gift of Dr Weiqun Li (Georgetown University). Transfected clones were selected by limited dilution cloning, in which cells were diluted to one cell per 5-mm cell culture well, and assessed for changes in PKC-δ activity by measuring kinase activity of calcium-independent PKC towards the PKC pseudo-substrate peptide (Upstate Biotechnology, Lake Placid, New York, USA [33,34]). Kinase-specific activity was calculated by subtracting basal activity from total catalytic activity and expressed as fmol/phosphate/min/mg [34].

Results

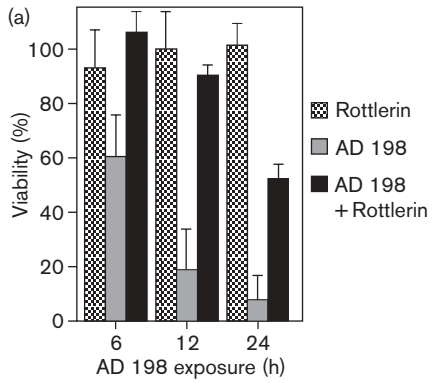
Blocking PCK-δ activity inhibits AD 198-induced cell kill

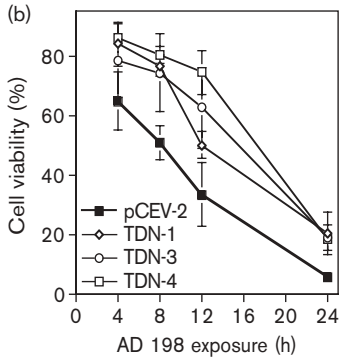
As we have shown previously, AD 198 induces rapid apoptosis in 32D.3 murine myeloid cells, with less than 20% of the initial cell population remaining viable within 12 h after a 1-h exposure to 5 μmol/l AD 198 [31] (Fig. 1a). The PKC inhibitor, rottlerin, at a concentration that selectively inhibits PKC-δ activity (10 μmol/l) [40], decreases cell sensitivity to AD 198, increasing cell viability to 80% after 12 h post-drug exposure and from 8 to 50% after 24 h. Previous reports have suggested, however, that rottlerin may have mitochondrial effects that occur independently of PKC-δ [41]. In order to confirm that specific inhibition of PKC-δ activity decreases 32D.3 sensitivity to AD 198, we transfected 32D.3 cells with the PKC-δK376R transdominant-negative PKC-δ expression vector. A representative clone, TDN-4, exhibits Ca^+-independent PKC activity that is reduced by 42% relative to control cells transfected with non-recombinant vector (pCEV-2). Reduced PKC activity corresponds to a delay in 50% cell kill from 8 to 18 h after AD 198 treatment. In contrast, reduced PKC activity had no significant effect on AD 288 cytotoxicity (data not shown). Together with previous studies using an array of PKC inhibitors, these results indicate that PKC-δ activity is required for AD 198-mediated cell kill. We have previously demonstrated that the mitochondria of untreated 32D.3 cells contain PKC-δ [31], suggesting that mitochondria are a likely target for AD 198.

AD 198 activates the PKC-δ holoenzyme to trigger mitochondrial depolarization and apoptosis

We next assessed the effect of PKC-δ inhibition on AD 198-induced mitochondrial membrane depolarization ($\Delta\psi_m$). Figure 2 shows the status of mitochondria membrane potential by staining with DePsipher, a fluorescent cationic dye which dimerizes within polarized mitochondria to yield a punctate red pattern, as shown in control (DMSO-treated) 32D.3 cells. Treatment of cells with 5 μmol/l AD 198 for 3 h diminishes punctate fluorescence and induces cell shrinkage in approximately 75% of the observed cells. Combined exposure of cells to 10 μmol/l rottlerin and 5 μmol/l AD 198 preserves punctate fluorescence, indicating the preservation of mitochondrial membrane potential through PKC-δ inhibition. Competition with ATP binding [40] allows rottlerin to inhibit the activity of both PKC-δ holoenzyme and the constitutively active 40-kDa catalytic subunit released by caspase-3 during apoptosis. To determine whether AD 198-induced $\Delta\psi_m$ is mediated through holoenzyme activation or results less directly from caspase-mediated cleavage of PKC-δ, 32D.3 cells were treated with 5.0 μmol/l AD 198 in combination with the

Fig. 1





PKC-δ activity

	Activity (fmol phosphate/min/mg)	Percent of control
PCEV-2	17.83	100.0
TDN-4	10.40	58.3
Fold difference	1.70 ± 0.06	

PKC-δ mediates AD 198-induced apoptosis. (a) 32D.3 cells were pre-treated for 2 h with either 10 μmol/l rottlerin, alone, or followed by 1-h 5 μmol/l AD 198 exposure and incubation in drug-free medium in the presence of rottlerin. Viability was determined by Trypan blue staining. Results represent the mean ± SEM of three independent determinations. (b) 32D.3 cells were transfected with either pCEV empty expression vector or a pCEV/TDN-PKCδ vector expressing the transdominant-negative PKC-δ isoform. PKC-δ activity was quantified as described in Materials and methods. AD 198 cytotoxicity in three clones was assessed by Trypan blue staining at the times indicated after a 1-h treatment with 5 μmol/l AD 198. Each point represents the mean of at least three independent determinations, each consisting of 300–500 cells per count.

pan-caspase inhibitor Z-VAD-FMK under conditions (200 μmol/l; 3 h) that inhibit PKC-δ cleavage [31] (Fig. 2). Inhibition of PKC-δ cleavage by Z-VAD-FMK fails to inhibit AD 198-induced $\Delta\psi_m$ as indicated by the loss of punctate fluorescence along with cell shrinkage. These results indicate that AD 198 activates PKC-δ holoenzyme to achieve mitochondrial depolarization. AD 198-mediated mitochondrial depolarization is not blocked by the expression of Bcl-2 or Bcl-X_L [31,32]. Since one inhibitory function of the anti-apoptotic Bcl-2 proteins is prevention of PTPC formation and activation [1], we further assessed the ability of AD 198 to trigger $\Delta\psi_m$ despite inhibition of PTPC activation (Fig. 2). CsA has been shown to bind to CyP-D [42], and inhibit both $\Delta\psi_m$ and cytochrome *c* release induced by a wide variety of stimuli [9,43]. Treatment of 32D.3 cells with 5 μmol/l CsA for 4 h had no effect on $\Delta\psi_m$ (not shown). Betulinic acid, a novel anti-tumor agent that induces apoptosis through direct interaction with mitochondria [4], induces $\Delta\psi_m$ in a manner that is inhibited by CsA treatment [1], as shown by the preservation of punctate fluorescence. However, 5 μmol/l CsA does not inhibit AD 198-induced $\Delta\psi_m$.

The release of cytochrome *c* often coincides with $\Delta\psi_m$ and is an essential component for caspase-3 activation [1]. AD 198 treatment of 32D.3 cells results in the rapid release of cytochrome, detectable by immunoblot analysis of cytosolic fractions after 2 h of drug exposure (Fig. 3a). Combined treatment with 10 μmol/l rottlerin inhibits cytochrome *c* release. Five μmol/l CsA treatment in combination with AD 198, however, did not inhibit cytochrome *c* release. Further, increasing concentrations of CsA up to 15 μmol/l failed to inhibit cytochrome *c* release by AD 198 (Fig. 3b). These results indicate that AD 198-mediated cytochrome *c* release requires PKC-δ activation, but does not require PTPC formation and opening.

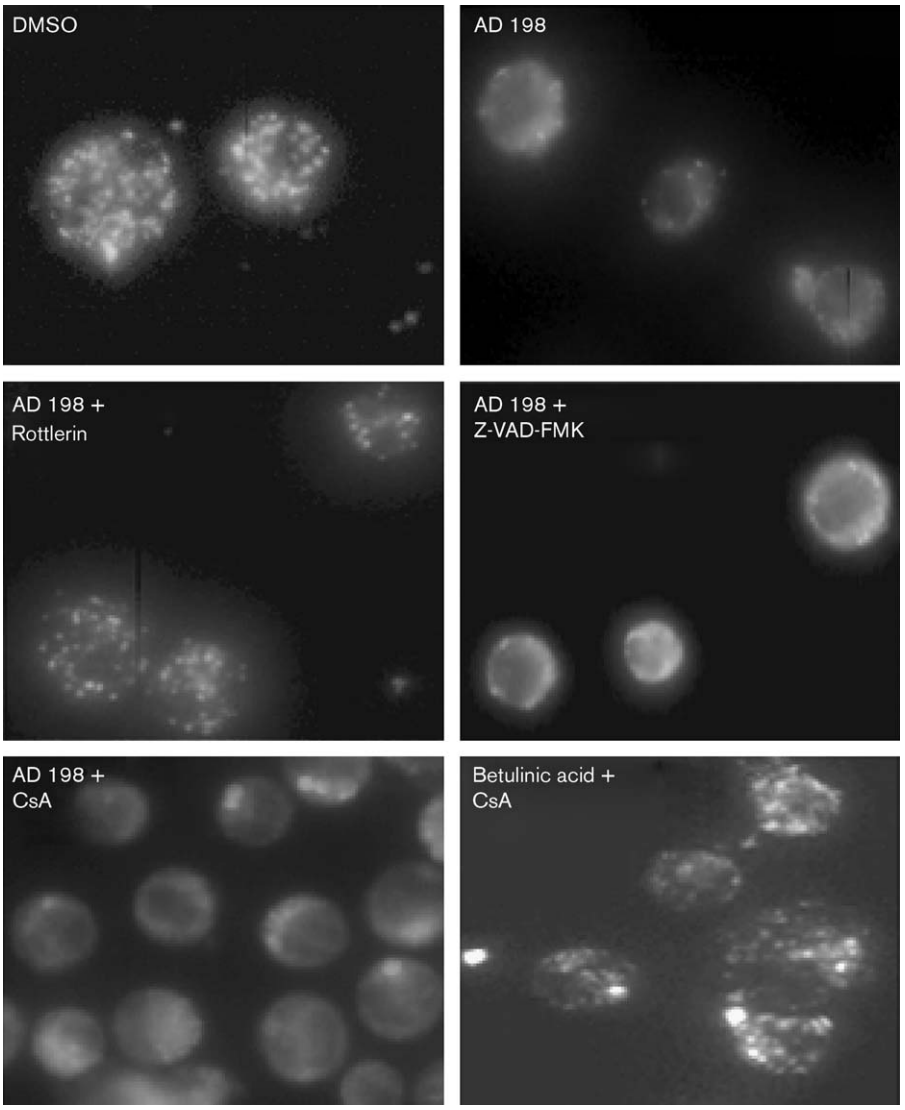
AD 198-induced mitochondrial depolarization is Ca^{2+}-independent

The independence of AD 198-mediated $\Delta\psi_m$ and cytochrome *c* release from PTPC functional status suggests that Ca^{2+} uptake may trigger $\Delta\psi_m$ through alternate mitochondrial transport [10–12] or does not play a role in AD 198-mediated mitochondrial responses. Consequently, intra- and extracellular Ca^{2+} was chelated with BAPTA-AM and EGTA, respectively, prior to AD 198 treatment to determine whether AD 198-mediated cell kill is Ca^{2+} dependent in 32D.3 cells (Fig. 4a). The Ca^{2+} ionophore A23187 induced greater than 90% cell kill within 1 h of treatment of 32D.3. BAPTA-AM/EGTA treatment, however, inhibited A23187 cytotoxicity, with less than 15% cell kill after 6 h beyond that observed with BAPTA-AM/EGTA alone, indicating effective Ca^{2+} chelation. Under these conditions, the rate of AD 198-mediated cell kill was unaffected by Ca^{2+} chelation. Further, BAPTA-AM/EGTA treatment also blocked cytochrome *c* release induced by A23187, but failed to block release by AD 198 or the closely related AD 445 (Fig. 4b), suggesting that AD 198-induced apoptosis does not require cellular Ca^{2+} flux.

Discussion

The results of our previous studies [31] and this present study indicate that AD 198 triggers mitochondrial-dependent apoptosis in 32D.3 murine myeloid cells through activation of PKC-δ holoenzyme. Further, the mechanism by which mitochondrial membranes undergo depolarization and promote cytochrome *c* release for

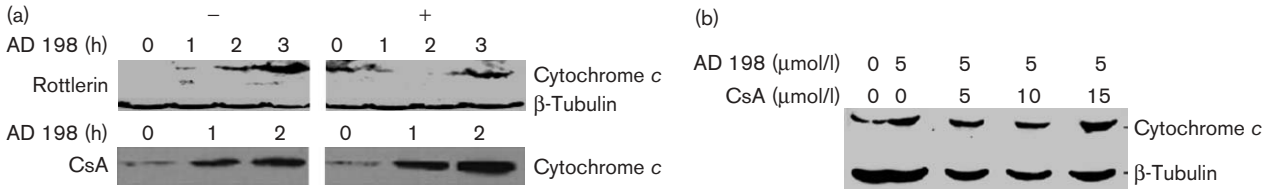
Fig. 2



Modulation of mitochondrial membrane potential by AD 198. 32D.3 cells were treated with either DMSO alone, 5 μmol/l AD 198, or 5 μmol/l AD 198 and either 10 μmol/l rottlerin, 200 μmol/l Z-VAD-FMK or 5 μmol/l CsA for 3 h, then stained with DePsipher for 20 min and observed by fluorescence microscopy. Alternatively, cells were treated with 15 μg/ml betulinic acid for 5 h or treated with 5 μmol/l CsA for 1 h followed by combined 5 μmol/l CsA and 15 μg/ml betulinic acid for 5 h. Cellular fluorescence distribution was observed by epifluorescence microscopy. The photomicrographs are representative of at least three independent determinations.

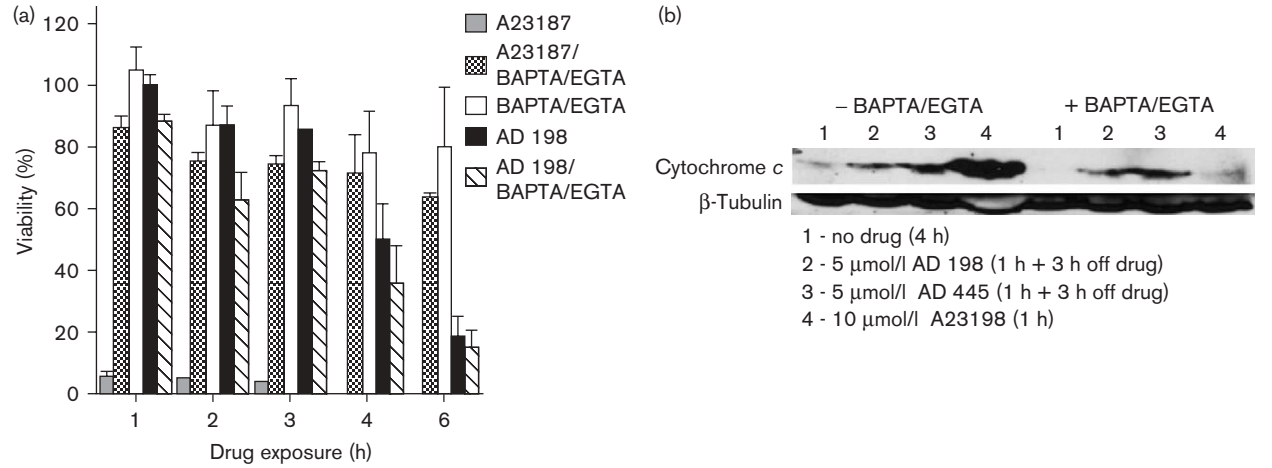
subsequent involvement in caspase activation is not dependent upon PTPC formation or Ca^{2+} flux and suggests a novel pathway for AD 198-induced apoptosis. PKC-δ has been shown previously to be a potent mediator of apoptotic signaling, both as a holoenzyme [15,16] and following caspase-mediated cleavage to yield a constitutively active catalytic subunit [44–46]. Further, the holoenzyme and cleaved catalytic fragment may coordinate their activities in a positive feedback loop mediated by caspase-3 action [18,47]. While blockade of caspase-3-mediated PKC-δ cleavage by Z-VAD-FMK inhibits late-stage AD 198-mediated apoptosis, as monitored by Trypan blue staining [31], the detection of both $\Delta\psi_m$ and cytochrome *c* release in the presence of Z-VAD-FMK indicates that PKC-δ cleavage is not a requisite step for mitochondrial recruitment following AD 198 treatment, but, rather, PKC-δ holoenzyme activity is sufficient and required. The requirement for PKC-δ activity for AD 198-mediated apoptosis is supported by the ability of rottlerin to inhibit $\Delta\psi_m$ and cytochrome *c* release induced by AD 198, and, consequently, caspase-3-mediated apoptotic events such as DNA endonucleolytic activity, poly(ADP-ribose) polymerase cleavage and PKC-δ cleavage [31]. The inability of rottlerin to inhibit

Fig. 3



Cytochrome *c* release by AD 198. (a) 32D.3 cells were exposed to 5 μmol/l AD 198 with or without 10 μmol/l rottlerin or 5 μmol/l CsA for the times indicated, then fractionated to isolate the cytosolic fraction as described in Materials and methods, subjected to SDS–PAGE and transferred to nitrocellulose. Filters were treated with anti-cytochrome *c* monoclonal antibody (1:250) for 2 h, followed by a 1-h treatment with 1:1000 dilution of horseradish peroxidase-conjugated goat anti-mouse second antibody. Proteins were detected by chemiluminescence. (b) 32D.3 cells were treated with AD 198, alone, or with the concentration of CsA indicated followed by combined treatment with CsA and AD 198 for 2 h. Cytosolic fractions were analyzed for cytochrome *c* content as described for (a). The immunoblots are representative of at least three independent determinations.

Fig. 4



Ca^{2+} chelation by BAPTA-AM/EGTA fails to inhibit AD 198 cytotoxicity or cytochrome *c* release. (a) 32D.3 cells were treated either with 5 μmol/l AD 198 or 10 μmol/l A23187 for 1 h and then incubated in drug-free medium for the times indicated. Alternatively, cells were treated with 25 μmol/l BAPTA-AM/3 μmol/l EGTA for 1 h prior to and during drug exposure, then during incubation in drug-free medium for the times indicated. Cell viability was monitored by Trypan blue staining. Results are the mean of three independent determinations. (b) Immunoblot analysis of cytochrome *c* release into cytosol was performed as described in Fig. 3.

$\Delta\psi_m$ triggered by AD 288 or apoptosis triggered by both AD 288 and DOX [31] suggests that mitochondrial recruitment by AD 288 and DOX does not require PKC-δ holoenzyme activity. Both AD 288 and DOX inhibit topoisomerase II, albeit through different mechanisms [37], and, consequently, trigger mitochondrial-dependent apoptosis through DNA damage/cell cycle arrest leading to p53-mediated expression of Bax. This would also be consistent with the ability of Bcl-2 and Bcl-X_L expression to inhibit both AD 288- and DOX-mediated apoptosis [31,32]. We have previously shown that AD 198, and the structurally and functionally similar AD 445, trigger apoptosis through a mechanism that is independent of DNA damage [32]. Consequently, AD 198 cytotoxicity is not affected by p53 dysfunction or Bcl-2/Bcl-X_L expression [31,32].

This latter observation poses the question of how AD 198 and, for that matter, AD 445 achieve PKC-δ-mediated $\Delta\psi_m$ and cytochrome *c* release in the presence of Bcl-2. Phorbol ester-mediated $\Delta\psi_m$ and cytochrome *c* release through PKC-δ holoenzyme activation is effectively blocked in LNCaP cells expressing Bcl-2 [16], yet AD 198 and AD 445 cytotoxicity remains unaffected by Bcl-2 expression in myeloid cells, MCF-7 breast carcinoma cells [31] and LNCaP cells (unpublished results). Multiple anti-apoptotic mechanisms have been ascribed to Bcl-2, including heterodimerization with pro-apoptotic Bcl-2 family members, the mediation of Apaf-1 binding to the mitochondria membrane surface, inhibition of PTPC opening and inhibition of Ca^{2+} release from the ER [15]. Our previous studies have indicated that Bcl-2 protein levels and phosphorylation status remain unchanged in 32D.3 cells following AD 198 treatment, suggesting that

AD 198 bypasses the anti-apoptotic effects of Bcl-2 rather than inhibits Bcl-2 expression and activation [31]. Consistent with these findings is the ability of AD 198 to trigger $\Delta\psi_m$ and cytochrome *c* release in the presence of CsA, which binds to CyP-D, blocks PTPC activity and, consequently, inhibits apoptosis by a variety of genotoxic agents [1]. Flavopiridol, a cyclin-dependent kinase inhibitor, has, likewise, been reported to trigger $\Delta\psi_m$ and apoptosis in HeLa and SW2 cells in a manner independent of the effects of Bcl-2 and the PTPC inhibitor, bongkrekic acid [48]. This action has been ascribed either to the caspase-8-mediated activation of a mitochondrial-independent apoptotic pathway or the ability of flavopiridol to directly induce PTPC opening [48]. 32D.3 cells are FADD deficient [31] and do not induce apoptosis in a mitochondrial-independent pathway. Thus, it is likely that $\Delta\psi_m$ and cytochrome *c* release are a direct effect of AD 198 treatment rather than a secondary feedback effect. The sesquiterpene, helenalin, circumvents the anti-apoptotic effects of Bcl-2, yet still triggers mitochondrial-dependent apoptosis in Jurkat T cells [49]. Cytochrome *c* release following helenalin treatment is caspase independent, whereas $\Delta\psi_m$ appears to be caspase dependent. AD 198-induced $\Delta\psi_m$ and cytochrome *c* release, however, are both caspase independent, based upon the inability of Z-VAD-FMK to block either event. Our results suggest that AD 198 triggers mitochondrial apoptotic events through a novel pathway initiated by PKC-δ holoenzyme activation. Since PKC-δ is a Ca^{2+}-independent isoform, the chelation of intracellular Ca^{2+} would still permit PKC-δ activation. Therefore, elucidation of the apoptotic signaling pathway activated by AD 198 requires the identification of mitochondrial PKC-δ targets. One target of current interest is PLS3, a mitochondrial member of a family of enzymes capable of translocating phospholipids between inner and outer layers of a lipid bilayer membrane [50]. Increased expression of PLS3 has been shown previously to enhance UV radiation-induced apoptosis, while inhibition of PLS3 blocks UV and drug-induced apoptosis [50,51]. PLS3 has also recently been shown to be a substrate for PKC-δ; phorbol ester-mediated phosphorylation of PLS3 via PKC-δ leads to mitochondrial-dependent apoptosis in HEK293 and HeLa cells [50]. Further, we have recently demonstrated that in HeLa cells, AD 198 can trigger PLS3-mediated $\Delta\psi_m$ and apoptosis through PKC-δ activation [52]. It is currently unknown whether PLS3-mediated apoptosis can occur independently of PTPC activation, Ca^{2+}-flux and the anti-apoptotic effects of Bcl-2 or whether PLS3 activation is just one of several apoptotic pathways triggered by AD 198. While PLS3 can be activated in a Ca^{2+}-dependent manner [50], it has been reported recently that the anesthetic agent, dibucaine, triggers phosphotidylserine translocation, $\Delta\psi_m$ and apoptosis in platelets in the absence of Ca^{2+} flux [53]. In summary, AD 198 is capable of triggering novel PKC-δ holoenzyme-dependent apoptotic signaling that is independent of PTPC pore activity and Ca^{2+} flux, and allowing circumvention of Bcl-2-mediated apoptotic inhibition.

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