Interaction of the Novel Anthracycline Antitumor Agent *N*-Benzyladriamycin-14-valerate with the C1-Regulatory Domain of Protein Kinase C: Structural Requirements, Isoform Specificity, and Correlation with Drug Cytotoxicity[1]

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Abstract

Anthracycline antibiotics like doxorubicin (DOX) are known to exert their antitumor effects primarily via DNA intercalation and topoisomerase II inhibition. By contrast, the noncross-resistant cytoplasmically localizing DOX analogue, *N*-benzyladriamycin-14-valerate (AD 198), only weakly binds DNA and does not inhibit topoisomerase II, yet it displays superior antitumor activity, strongly suggesting a distinct cytotoxic mechanism. In recent modeling studies, we reported a structural similarity between AD 198 and commonly accepted ligands for the C1-domain of protein kinase C (PKC), and we hypothesized that the unique biological activity of AD 198 may derive, in part, through this kinase. Consistent with this hypothesis, the present biochemical studies demonstrate that AD 198 competes with [^{3}H]phorbol-12,13-dibutyrate ([^{3}H]PDBu) for binding to phorbol-responsive PKC isoforms, the isolated C1b domain of PKC-δ (δ C1b), and the nonkinase phorbol ester receptor, β2-chimaerin. In NIH/3T3 cells, AD 198 competitively blocks PKC activation by C1-ligands. Importantly, neither DOX nor *N*-benzyladriamycin, the principal AD 198 metabolite, inhibits basal or phorbol-stimulated PKC activity or appreciably competes for [^{3}H]PDBu binding. In CEM cells, structure activity studies with 14-acyl congeners indicate that the rapid induction of apoptosis correlates with competition for [^{3}H]PDBu binding, strongly implicating phorbol-binding proteins in drug activity. Collectively, these studies support the conclusion that AD 198 is a C1-ligand and that C1-ligand receptors are selective drug targets. These studies provide the impetus for continuing efforts to understand the molecular basis for the unique biological activity of AD 198 and provide for the design of analogues with improved affinity for C1-domains and potentially greater antitumor activity.

Introduction

Introduced into the clinic >25 years ago, the antitumor antibiotic DOX[3] (Adriamycin) remains one of the most widely used drugs in the management of patients with cancer (1). Unfortunately, the curative potential of this drug is compromised significantly by the well-recognized risk of cumulative dose-related cardiomyopathy and also by the development, through several mechanisms, of drug resistance (2). These several considerations have provided an impetus for the search for DOX derivatives with superior therapeutic properties. Although such efforts elsewhere have given rise to various experimental agents, some of which, *e.g.,* epirubicin and idarubicin, have indeed progressed to clinical approval, these agents generally offer only minor changes from DOX in terms of pharmacology. All appear to produce their cytotoxic effects primarily through mechanisms typical of DOX, namely DNA binding and inhibition of DNA topo II (1–3). They are, therefore, similarly cardiotoxic and largely subject to the various well-documented cellular drug resistance mechanisms (*e.g.,* P-glycoprotein, multidrug resistance protein, and altered topoisomerase enzyme), and, for this reason, none appears to provide compelling advantages over DOX for the treatment of systemic disease (4).

In connection with a broad-based program on DOX pharmacology, our laboratories have been responsible for the discovery and development of several mechanistically atypical, highly active noncardiotoxic analogues of the parental antibiotic. One of these, the DNA-nonbinding AD 32, has

Received 3/21/02; accepted 3/27/02.
[1] Supported by CA 44890 (to T. W. S.), CA 74197 (to M. G. K.), ACS RPG-97-092-01-CNE (to M. G. K.), NIH Medical Student Research Fellowship T35 DK07405-11 (to J. B. R.), and the Computational Research on Materials Institute at the University of Memphis.
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[3] The abbreviations used are: DOX, doxorubicin; AD 198, *N*-benzyladriamycin-14-valerate; AD 32, valrubicin, *N*-trifluoracetyladriamycin-14-valerate; AD 288, *N*-benzyladriamycin; PKC, protein kinase C; PDBu, phorbol-12,13-dibutyrate; PMA, phorbol 12-myristate 13-acetate; DAG, diacylglycerol; DOG, dioctylglycerol; cPKC, classical PKC isoform; topo II, DNA topoisomerase II; GST, glutathione *S*-transferase; PS, phosphatidylserine; HPLC, high-performance liquid chromatography; PC, phosphatidylcholine; PEG, polyethylene glycol.

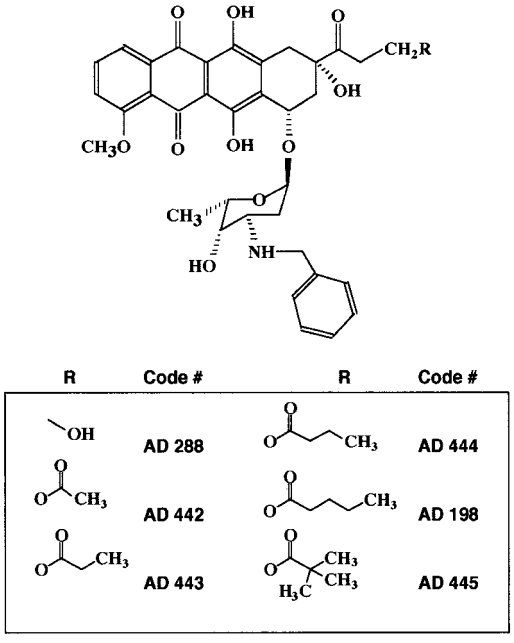

Fig. 1. Anthracycline analogues.

recently received Food and Drug Administration approval for use as a topical agent in the treatment of refractory superficial carcinoma of the bladder (5). The present report concerns another highly active noncardiotoxic DOX analogue from our program, AD 198 (Fig. 1), mechanistic studies with which indicate PKC to be a putative target for its cytotoxic action.

AD 198 produces DNA strand breaks visible on alkaline elution, but in contrast to DOX, it only weakly binds isolated DNA and is itself virtually devoid of topo II inhibitory activity (reviewed in Ref. 4). Moreover, although DOX localizes in the nucleus, AD 198 localizes almost exclusively in the cytoplasm, with no detectable nuclear accumulation. Notwithstanding the lack of a significant interaction with usual DOX targets, AD 198 is a potent nonphase-specific cytotoxic agent with superior experimental antitumor activity to DOX (6). Significantly, it is also capable of circumventing multiple forms of DOX resistance (7–9). Therefore, it would appear that the modifications made to the antibiotic structure in its semisynthetic conversion into AD 198 have conferred on the analogue a mechanism of cytotoxicity different from DOX.

Our hypothesis that PKC may be a cellular target for AD 198, and that this may be responsible for the cytotoxic effects of this novel agent, evolved from several considerations. PKC is a family of lipid-dependent serine/threonine kinases involved in numerous cellular processes (10, 11). More specifically, the role of PKC isoforms in the regulation of cellular proliferation and apoptosis makes this family of kinases an attractive target for cancer chemotherapy (12, 13). Chuang *et al.* (14) previously identified PKC as a possible target for AD 32, a closely related structural congener of AD 198; PKC inhibition by AD 32 was suggested to be specific for the 14-valerate substitution, as evidenced by the weak

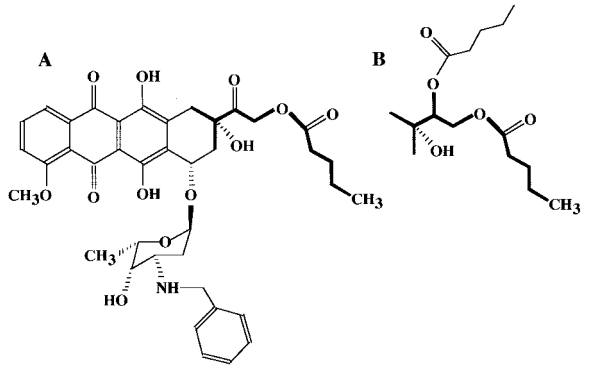

Fig. 2. Isosteric similarity between AD 198 (*A*) and DAG (*B*).

inhibitory effects both of DOX and another congener lacking this substitution. A close investigation of the three-dimensional structure of the PKC agonist DAG shows evidence of isosteric similarity with the 14-valerate side-chain of AD 198 (Fig. 2). Moreover, Wang *et al.* (15) reported on common structural features between anthracyclines and ligands for the C1-domain of PKC. We extended recently the observation regarding anthracyclines and PKC to AD 198 by conducting docking studies of this drug with the phorbol-binding C1b domain of PKC-δ (16). In the present studies, we have characterized the interaction of AD 198 with various PKC isoforms *in vitro* to confirm our hypothesis regarding a specific interaction with the C1-domain of this kinase.

Materials and Methods

Chemicals. Hydrochloride salts of the DOX congeners (see Fig. 1) were prepared as described previously (17, 18). PC, PS, PEG, and protamine chloride were purchased from Sigma Chemical Co. (St. Louis, MO). [γ-^{32}P]-ATP (3,000 Ci/mmol) and [20-^{3}H]-PDBu (35,320 dpm/pmol) were purchased from Amersham (Arlington Heights, IL). PDBu, PMA, GF109203X, and DOG were obtained from Calbiochem (San Diego, CA). The peptide substrate derived from the pseudosubstrate of PKC-α was from Life Technologies, Inc. (Rockville, MD).

Rat Brain PKC and Recombinant Proteins. Recombinant human PKC-δ was from Pan Vera (Madison, WI). A mixture of classical PKC isoforms was isolated from rat brain, as described previously (19). It has been shown previously that rat brain-derived PKC preparations have biochemical properties nearly identical to those of the isolated classical isoforms (20). The PKC-δ C1b domain and β2-chimaerin GST fusion proteins were expressed and isolated as described previously (21, 22). For PKC-ζ, an *Eco*RI-*Eco*RI fragment comprising full-length cDNA for mouse PKC-ζ was subcloned into the GST-baculovirus expression vector pACG2T (PharMingen; Refs. 23 and 24). A recombinant baculovirus for GST-PKC-ζ was generated using standard techniques. Sf9 cells were infected with GST-PKC-ζ at a multiplicity of infection of 10 pfu/cell for 48 h. GST-PKC-ζ was purified from Sf9 cells using glutathione Sepharose beads (Pharmacia). PKC-ζ and the PKC-δ C1b domain were

released from GST with thrombin treatment using the supplied protocol (Pharmacia).

Preparation of Drug-Lipid Mixtures. By their nature, AD 198 and the other lipophilic DOX analogues are difficult to formulate in the aqueous buffer systems commonly used for *in vitro* PKC assays. In the present studies, this difficulty was avoided by preparing the lipophilic agents as mixed vesicles with a constant 20% PS and 60–80% PC, with the test agent representing the balance of the 100 μM total lipid. Mixed drug-lipid vesicles were prepared essentially as described for other lipophilic modulators of PKC, *e.g.,* DAG (25). Briefly, drugs were dissolved in chloroform:methanol (10:1, volume for volume) and combined with phospholipid solutions as supplied by the manufacturer. Organic solvents were removed under a stream of nitrogen with subsequent drying under vacuum for 2 h. The resulting lipid-drug films were hydrated with the indicated buffer, either 20 mM HEPES or 50 mM Tris, in a water bath (at 37°) for 30 min. Small unilamellar vesicles were generated by sonication of hydrated lipid mixtures for 10 s. The PS concentration was maintained constant at 20 mol % by omission of a molar equivalent of PC as the drug concentration was increased. The propensity for anthracyclines to self-aggregate at high drug:lipid ratios prevented the preparation of mixed vesicles with interfacial drug concentrations exceeding 20 μM (20 mol %).

Kinase Assay. Cofactor-dependent or -independent kinase activity of PKC toward the PKC-α pseudosubstrate peptide (ser[25]; Life Technologies, Inc., Grand Island, NY) or protamine in the presence of DOX congeners (0–20 μM) was measured essentially as described by Leventhal and Bertics (19), with minor modifications. Briefly, PKCs (5–25 ng), 100 μM total lipid (20 mol % PS: 60–80% PC), 10 μM $CaCl_2$, 10–25 μM PKC-α pseudosubstrate peptide, or 0.2 mg/ml protamine chloride and the indicated concentrations of PDBu and drugs were preincubated for 2 min at 37° or at room temperature, for the rat brain preparation or recombinant isoforms, respectively. The kinase reaction was initiated by the addition of a small volume of [^{32}P]-ATP/Mg solution [final incubation volume of 50 μl containing 100 μM ATP (200–500 cpm/pmol) and 10 mM $MgCl_2$]. After 10 min, the kinase reaction was terminated by the addition of 2% phosphoric acid (30 μl), and the solution was cooled on ice for 5 min. After the spotting of a 70-μl aliquot of the cooled reaction mixture onto 2.5-cm P81 phosphocellulose filters (Whatman), the filters were washed (3 × 300 ml) with 0.5% H_3PO_4, then with methanol (100 ml), and were air dried. [^{32}P]-Phosphate incorporation was measured by scintillation counting. When the kinase activity of PKC-δ or PKC-ζ was measured, the assay components were identical to those described above, except that 1 mM EGTA was substituted for calcium in the reaction mixture. PDBu was excluded from the reaction mixture when either PKC-ζ or phosphorylation of protamine was assayed.

Kinase Assay with Cell Lysates. NIH/3T3 cells were grown on 60-mm tissue culture plates in DMEM media with 10% fetal bovine serum, with subconfluent cells passaged every 3rd day. Subconfluent NIH/3T3 cells were exposed to 1% DMSO vehicle, AD 198, or other indicated test agents for 30 min followed by a 15-min coexposure with 10, 100, or 1 μM PMA. Plates were washed once with 10 ml of ice-cold PBS, and cells were harvested by scraping into a buffer containing 20 mM HEPES, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 mM NaF, and 1 mM Na_2VaO_3. Cells were disrupted by sonication, and total lysates were used in the kinase assay essentially as described above, except that a final volume of 25 μl was used.

[^{3}H]PDBu Binding. Binding studies with [^{3}H]PDBu were carried out using the PEG precipitation method as described by Sharkey and Blumberg (26), with minor modifications. Briefly, rat brain PKC, recombinant PKC-δ, PKC-δ C1b, or GST-β2-chimaerin was combined with 1 mg/ml bovine gamma globulin, 50 mM Tris (pH 7.4), 0.3–20 μM test agent, 5 nM [^{3}H]PDBu, and 100 μM phospholipid (20% PS: 60–80% PC, as described above) in microcentrifuge tubes in a final volume of 250 μl. The resulting mixture was incubated for 30 min at room temperature and cooled on ice for 5 min, and the protein was precipitated by the addition of 200 μl of 35% PEG. When PKC-δ, PKC-δ C1b, or GST-β2-chimaerin were assayed, the incubation mixture included 1 mM EDTA. The incubation mixture was further chilled for 15 min, followed by a 15-min centrifugation (12,500 rpm at 4°) in a Beckman 12 microcentrifuge. A 100-μl aliquot of supernatant was used to determine the free PDBu concentration, and the remaining supernatant aspirated. After removal of the tube tip with a hot scalpel, the protein pellet therein was first dissolved in NaOH (0.5 ml, 0.2 M), then resuspended in scintillation fluid for counting. K_i values were determined as described previously (24).

[^{3}H]PDBu Binding in Cell Lysates. After the exposure of human CEM lymphoblastic leukemia cells to AD 198 (0–20 μM; 10^6 cells/ml) for 1 h, cells were washed with ice-cold PBS and harvested in 50 mM Tris (pH 7.4) containing 1 μg/ml leupeptin, and cell membranes were disrupted by sonication (15 s). Aliquots (50–150 μl) of the cell lysates were added to 50 mM Tris buffer containing 1 mM $Cacl_2$ or 1 mM EGTA and [^{3}H]PDBu (35,320 dpm/pmol in 20 mg/ml γ globulin) for a final volume of 250 μl. The resulting mixture was incubated at room temperature for 30 min before protein precipitation. Thereafter, samples were processed exactly as described above.

***In Vitro* Cytotoxicity Assay.** The respective DOX congeners (0–20 μM), dissolved in DMSO (final concentration: <1%) were added to human CEM lymphoblastic leukemia cells in log growth phase (10^6 cells/ml) for 1 h. Cells were then pelleted, washed with saline, and replated in microtiter plates using fresh media for an additional 7-h incubation. At this time, cytotoxicity was assessed by standard tetrazolium dye assay. In parallel studies, cellular drug accumulation and integrity were determined in extracts (ethyl acetate/1-propanol) of duplicate cell pellets by a reversed-phase HPLC/fluorescence assay system (27).

Results

Fig. 3, *A–C* shows the interactions of AD 198 and a group of structurally related anthracyclines with the kinase activity of a highly purified preparation of rat brain PKC. In these assays, AD 198 blocked the phosphoryl-transfer activity of rat brain PKC toward a peptide substrate derived from the

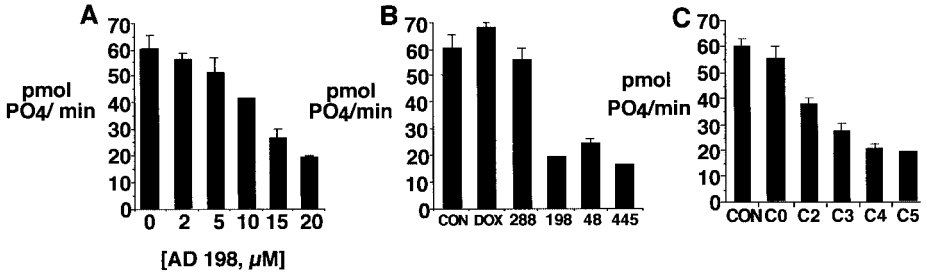

Fig. 3. A–C, inhibition of rat brain PKC kinase activity by AD 198 and congeners. In *A,* rat brain PKC (25 ng) was incubated with 5 nM PDBu and 10 μM $CaCl_2$ and increasing concentrations of AD 198 in mixed vesicles containing 20% PS and 60–80% PC. Kinase activity was measured as described in "Materials and Methods." Kinase activity in the absence of PDBu was 17.7 +/− 2.2 pmol of phosphate/min and was unaffected by AD 198. Kinase activity in the presence of 5 nM PDBu was 59.2 +/− 6 pmol of phosphate/min. In the absence of cofactors, activity was <10% of the total. *Bars,* SD of three independent experiments each carried out in duplicate. In general, duplicate determinations differed by <10%. *B,* rat brain PKC (25 ng) was incubated with 20 μM (20 mol %) of the indicated test drug (see Fig. 1), and phosphate transfer activity was measured as above. *Bars,* SD of three independent experiments each carried out in duplicate. *C,* kinase activity of rat brain PKC was measured as above in the presence of 20 μM (20 mol %) of the indicated *N*-benzyl compound (*CON,* control; *C0,* AD 288; *C2,* AD 442; *C3,* AD 443; *C4,* AD 444; *C5,* AD 198). Results are the mean of duplicate determinations and are representative of two to three independent experiments.

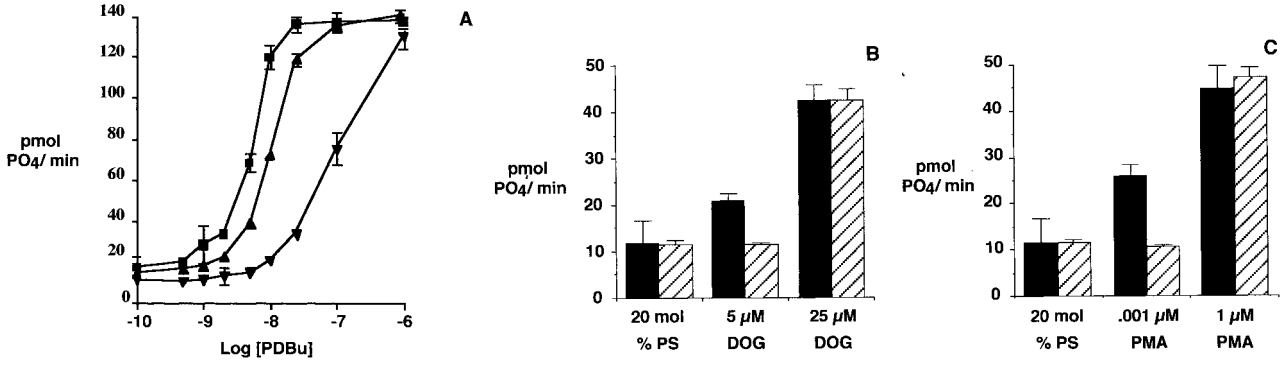

Fig. 4. In *A–C,* inhibition of PKC by AD 198 is competitive with ligands for the phorbol ester-binding domain. In *A,* rat brain PKC (25 ng) was incubated with 0 (■), 10 (♦), or 20 (◆) μM (0–20 mol %) AD 198 and increasing concentrations of PDBu as indicated, and PO_4 transfer was measured as described in "Materials and Methods." *Bars,* the range of duplicate determinations from a single experiment. A second experiment gave comparable results. Rat brain PKC (25 ng) was incubated with 0 (*solid bars*) or 20 μM (*hatched bars*) AD 198 and the indicated concentration of DOG (*B*) or PMA (*C*), as described above.

PKC-α pseudosubstrate sequence (Fig. 3*A*), whereas DOX and AD 288, the principal biotransformation product of AD 198, were without activity in this assay system (Fig. 3*B*). Moreover, adriamycin-14-valerate, which contains the valerate substituents but lacks the benzyl substitution on the 3′-amino group of the daunosamine sugar (Fig. 1), had inhibitory activity against rat brain PKC comparable with that of AD 198 (Fig. 3*B*). Furthermore, for a limited series of 14-acyl DOX congeners, the ability of these agents to inhibit rat brain PKC correlated with an increase in 14-acyl chain length up to the C5 valerate substitution (Fig. 3*C*). Of note, the hydrolysis-resistant homologue of AD 198, *N*-benzyladriamycin-14-pivalate, demonstrated PKC inhibitory activity similar to that of AD 198 (Fig. 3*B*), suggesting the importance of both hydrophobicity and chain length of the acyl substituents.

Maximal inhibition of rat brain PKC by AD 198 (Fig. 3*A*) corresponded to the level of activity observed in the absence of PDBu, suggesting that AD 198 may specifically block the activation of PKC by this ligand. Indeed, the data in Fig. 4 demonstrate that AD 198 shifted the PDBu dose-response curve for kinase stimulation to the right and that inhibition of PKC by AD 198 was surmountable by increasing the concentration of PDBu. As shown in Fig. 4, *B* and *C,* similar results were obtained with the potent phorbol ester, PMA, and with DOG, an analogue of the endogenous PKC activator, DAG. Thus, it appears that AD 198 specifically blocks the activation of PKC by ligands for the C1-regulatory domain.

The competitive behavior of AD 198 cannot be described by simple Michaelis-Menton kinetics. As shown in Fig. 4*A,* a 2-fold increase in the concentration of AD 198 results in a much larger shift in the apparent K_D for PDBu than would be predicted from classic Michaelis-Menton kinetics. This non-ideal behavior may reflect differential interactions with the individual C1-domains or, alternatively, the complexity of the assay system. With regard to the latter, the apparent cooperativity that arises from phase changes, *i.e.,* from the aqueous to lipid surface in vesicle-based assay systems (28), and the influence of surface-dilution phenomena may complicate kinetic analysis in a lipid-vesicle-based assay system (29). Rather than address these complex kinetic issues here, we chose instead to investigate the hypothesis that AD198 interacts with the phorbol binding domain with a simplified assay system using radioligand-binding techniques under equilibrium conditions, where K_i values could be more readily

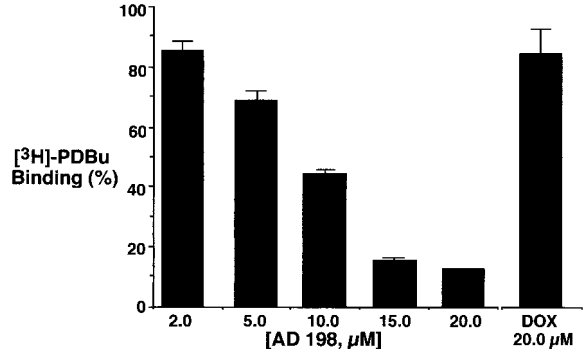

Fig. 5. AD 198 inhibits binding of [^{3}H]PDBu to rat brain PKC. Rat brain PKC (25 ng) was incubated with 5 nM [^{3}H]PDBu and increasing concentrations of AD 198 as indicated. Bound [^{3}H]PDBu was measured as described in "Materials and Methods." Specific binding was 400 pmol or 14,237 dpm. Nonspecific binding was <10% of the total bound. *Bars,* SD from the three independent experiments, each carried out in triplicate.

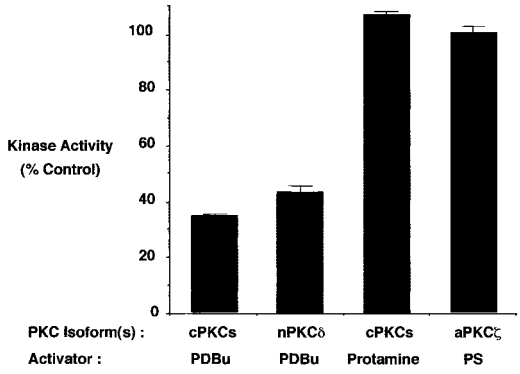

Fig. 6. Isoform and cofactor specificity of PKC-inhibition by AD 198. Phosphate transfer for each PKC isoform and condition indicated was measured as described in "Materials and Methods" in the presence of 60 mol % PC, 20 mol % PS, and 20 mol % AD 198. Rat brain PKC, representative of the cPKCs, and novel PKC-δ were activated by 5 nM PDBu. $CaCl_2$ (10 μM) was present in the reaction mixture for rat brain PKC and replaced with 1 mM EDTA for PKC-δ and PKC-ζ. PDBu was excluded from the reaction mixture when phosphorylation of protamine or the activity of PKC-ζ was measured.

determined. Using this approach, the competitive interaction between AD 198 and PDBu was investigated by competition-binding studies using [^{3}H]PDBu as a probe (Fig. 5). In these studies, AD 198 inhibited the specific binding of [^{3}H]PDBu to rat brain PKC in a dose-dependent manner, with an IC_{50} of 9 μM (K_i = 2.8 μM). Under the same assay conditions, DOX (20 μM) inhibited <20% of [^{3}H]PDBu specifically bound to PKC. In this concentration range, the relative inactivity of DOX for both kinase inhibition and for inhibition of [^{3}H]PDBu binding is consistent with a previous study by Nadatake *et al.* (30).

The data obtained with the rat brain PKC preparation (Figs. 3–5), which are representative of the activity of the classical PKC isoforms, suggest an interaction of AD 198 with the phorbol/DAG-binding site of PKC. If this were indeed true, such an interaction would be specific for the phorbol/DAG responsive novel and classic PKC isoforms. To test this hypothesis, the activity of AD 198 was assayed against the kinase activity of recombinant preparations of PKC-δ and PKC–ζ, which are representative of the novel and atypical isoforms, respectively (Fig. 6). As with the cPKC mixture from rat brain, AD 198 inhibited PDBu-stimulated kinase activity of novel PKC-δ but had no effect on the activity of the phorbol-insensitive PKC-ζ. Protamine is a highly basic peptide that is phosphorylated by PKC through a mechanism insensitive to the presence of C1-ligands or other cofactors (19). As shown in Fig. 6, AD 198 does not inhibit the phosphorylation of protamine by rat brain PKC. Therefore, it is unlikely that AD 198 interferes directly with the phosphorylation of substrates but rather interferes with cofactor regulation, presumably through the NH_2-terminal regulatory domain. The specificity of AD 198 for the regulatory domain of PKC is further supported by the lack of an effect of AD 198 on the catalytic subunit of PKC prepared from rat brain PKC by limited proteolysis (data not shown).

Binding of phorbol esters and DAG to novel and classical PKC isoforms is conferred by a 50–51 amino acid sequence within their NH_2-terminal C1-domains (31). The C1-domains of the novel and classical PKCs occur as a tandem repeat, C1a and C1b, respectively, of this conserved sequence. However, phorbol ester binding can be reconstituted with a peptide containing a single copy of the consensus sequence derived from the C1-domains of PKC (32). To determine whether AD 198 interacts specifically at the C1-domain, binding studies were carried out with a 50 amino acid peptide from the C1b domain of PKC-δ (δC1b) that binds [^{3}H]PDBu with high affinity (21). As shown in Fig. 7*A,* AD 198 inhibited specific [^{3}H]PDBu binding to the isolated C1b of PKC-δ (K_i = 2.7 $\pm$ 0.2 μM) in an identical manner to that observed for intact PKC-δ (K_i 2.5 $\pm$ 0.2 μM). Under these assay conditions, and consistent with the earlier rat brain cPKC studies, DOX (20 μM) inhibited <20% of specific [^{3}H]PDBu binding to either intact PKC-δ or to the δC1b (data not shown).

At present, members of the PKC superfamily constitute the majority of known phorbol ester receptors. However, additional proteins without kinase domains that have functional or "typical" C1-domains have been identified (33). One of these proteins, β2−chimaerin, binds phorbol esters with high affinity (22). To confirm the data regarding the direct interaction of AD 198 with C1-domains, the effect of this compound on [^{3}H]PDBu binding to β2−chimaerin was evaluated. As observed for the PKC isoforms and the isolated δC1b domain, AD 198 also inhibited [^{3}H]PDBu binding to β2-chimaerin in a dose-dependent manner with a K_i of 1.5 $\pm$ 0.2 μM (Fig. 7*B*).

To determine whether or not AD 198 interacts with PKC in intact cells in a similar manner to that observed *in vitro,* we investigated the activity of PKC in NIH/3T3 cells pretreated with AD 198 followed by PMA stimulation (Fig. 8). Treatment of NIH/3T3 cells with PMA resulted in the dose-dependent stimulation of phosphoryl-transfer activity toward the PKC-α pseudosubstrate peptide, and this increase in activity could be inhibited completely by the addition of 1 μM GF109203X, a potent and selective catalytic inhibitor of PKC (Fig. 8*A*). As observed *in vitro* with purified PKC preparations, pretreatment of NIH/3T3 cells with AD 198 blocked the stimulation of kinase activity seen with PMA, and, furthermore, blockade of

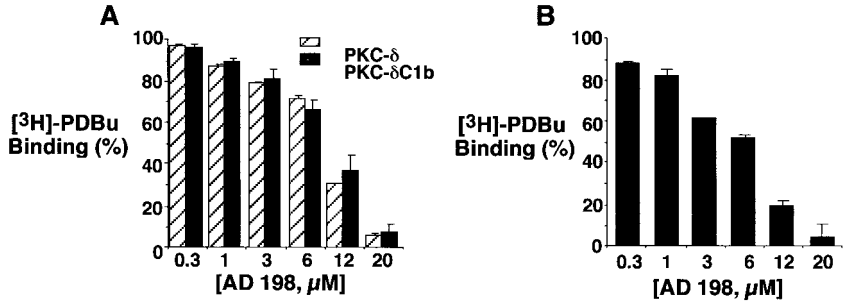

Fig. 7. In *A* and *B,* AD 198 inhibits [^{3}H]PDBu binding to recombinant PKC-δ, the isolated PKC-δC1b, and a GST-β2-chimaerin fusion protein. Recombinant PKC-δ (10 ng), isolated PKC-δ C1b (1 nM; *A*), or GST-β2-chimaerin fusion protein (*B*) were incubated with 5 nM [^{3}H]PDBu and increasing concentrations of AD 198 as described in "Materials and Methods." Total binding was ~6000, 9000, and 5000 dpm for PKC-δ, the PKC-δ C1b, and β2-chimaerin, respectively. Nonspecific binding, measured in the presence of 30 μM cold PDBu, was <20% of the total bound. *Bars,* SD of triplicate determinations from a representative experiment. Analogous results were obtained in at least two additional independent experiments with each phorbol-binding protein.

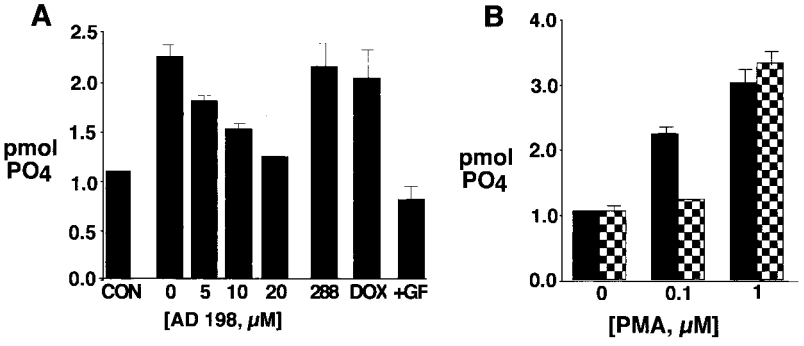

Fig. 8. In *A* and *B,* AD 198 competitively blocks PKC activation by PMA in NIH/3T3 cells. NIH/3T3 cells were treated with 0–20 μM AD 198, 20 μM DOX, or 20 AD 288 for 15 min, followed by an additional 15-min exposure to 100 nM PMA (*A*) or with 0, 0.1, or 1 μM PMA (solid bars)+ 20 μM AD 198 (hatched bars; *B*). In *A,* +GF indicates the effect produced by 1 μM GF109203X on phorbol-stimulated (100 nM) kinase activity. Cells were washed with ice-cold PBS and harvested into lysis buffer, and the phosphoryl-transferase activity of lysates toward the PKC-α pseudosubstrate peptide was measured as described in "Materials and Methods." Results are representative of three to four independent experiments. *Bars,* the range of duplicate determinations from a single experiment.

kinase stimulation was surmountable by a 10-fold increase in PMA concentration (Fig. 8*B*). Importantly, as also observed *in vitro,* both AD 288 and DOX were without effect in this assay system.

The CEM human acute lymphoblastic leukemia cell line has been used extensively in our laboratories to evaluate the cytotoxic effects of AD 198 and related anthracyclines. To examine whether the novel effects of AD 198 and the other 14-*O*-acyl DOX analogues on PKC, demonstrated above, correlate with the biological activity of these agents, a series of studies were conducted with the CEM cell line, using short-term (1 h) drug exposure. Initially, we attempted to measure the effects of AD 198 on PKC activity in CEM cell lysates; however, unlike in the studies using NIH/3T3 cells, we were unable to consistently measure kinase stimulation by PMA. However, we were readily able to measure [^{3}H]PDBu binding in these cells. After exposure to AD 198 (0–20 μM), cells were either lysed, and the inhibition of [^{3}H]PDBu binding was assessed (Fig. 9*A*), or drug cytotoxicity was determined in whole cells at 8 h by standard tetrazolium dye assay (Fig. 9*B*). Inhibition of [^{3}H]PDBu binding by AD 198 in CEM cell lysates directly parallels the data obtained with rat brain PKC (Fig. 5) and once again confirms the absence of an effect for either DOX or AD 288. Of significance, the cytotoxicity of AD 198 occurs over the same concentration range (5–12.5 μM), as does the inhibition of phorbol binding. This correlation between drug cytotoxicity and PKC effects appears also to extend to the remaining 14-*O*-acyl DOX analogues whose biological activity and *in vitro* biotransformation are shown in Table 1. An increase in length of the 14-*O*-substitution from a C2 (acetate) to a C5 (valerate) correlates with increasing potency (equal cellular drug accumulation, determined by HPLC analysis) in producing rapid (<8 h) cell death. Condensed and fragmenting nuclei consistent with apoptosis were evident in cells stained with bisbenzamide and examined with fluorescence microscopy at 4 h (data not shown). Thus, the relationship between the structure of the 14-*O*-acyl substituents and drug cytotoxicity in cell culture correlates well with that observed for competition with [^{3}H]PDBu binding and PKC inhibitory activity of these analogues *in vitro* (Figs. 3, *B* and *C*).

Discussion

Although AD 198 is structurally related to DOX, it has been amply evident since its discovery that this novel lipophilic

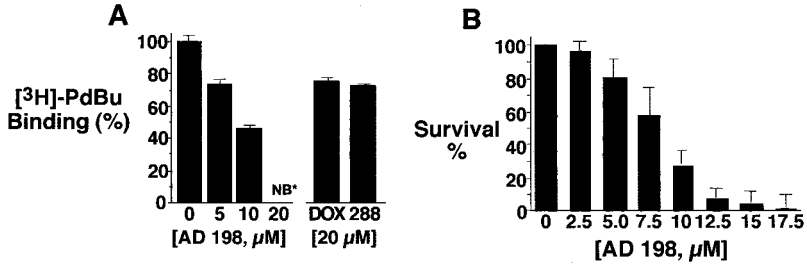

Fig. 9. In *A* and *B,* inhibition by AD 198 of [^{3}H]PDBu binding in CEM cell lysates correlates with cytotoxicity. After a 1-h incubation of AD 198 (0–20 μM) with human lymphoblastic leukemia cells, the drug effects were assayed as described in "Material and Methods," either by measuring the inhibition of [^{3}H]PDBu binding to a resulting cell lysate preparation (*A*) or by determining cell survival at 8 h using a standard tetrazolium dye assay (*B*). Data represent the mean ± SD for three (*A*) or four (*B*) independent experiments, each conducted in triplicate. NB*, no detectable binding of PDBu.

Table 1 Comparative cytotoxicity and metabolic stability of AD 198 and other 14-*O*-acyl anthracycline congeners in human CEM lymphoblastic leukemia cells

N-Benzyladriamycin-congener (14-*O*-acyl substitution)	IC_{50} (μM) for 1 h Drug exposure	% Intact drug at 1 h	% Intact drug at 8 h
AD 288 (C0, H)	>20[a]	100	100
AD 442 (C2, Acetate)	>20[b]	86	57
AD 443 (C3, Propionate)	14.0 ± 0.6	91	65
AD 444 (C4, Butyrate)	9.3 ± 0.5	90	65
AD 445 (C5, Pivalate)	8.1 ± 1.1	100	96
AD 198 (C5, Valerate)	7.7 ± 1.4	90	56

[a] 85 ± 6% survival at 20 μM.
[b] 75 ± 15% survival at 20 μM.

analogue possesses many properties that are atypical of the parental antibiotic. The fact that AD 198 localizes in the cytoplasm of cells yet can produce early DNA damage and circumvent DOX resistance mechanisms (6, 7) has always indicated a unique mechanism of action for 14-acyl drug congeners (4). Furthermore, short-term exposure of cells to 5 μM AD 198 results in significant membrane blebbing, an effect not seen with DOX at similar or higher concentrations (6). For the aforementioned reasons, we considered the PKC family as a potential target for AD 198 action, and the present studies were therefore undertaken to begin correlating the structure of AD 198 and its congeners with their effects on this important kinase family.

The structure activity studies reported here clearly demonstrate that AD 198, but neither AD 288 nor DOX, inhibits the classical or novel isoforms of PKC. Early reports suggested that DOX inhibited PKC (34–36). However, subsequent investigations, using refined assay conditions similar to those of the present study, demonstrated that the effect of DOX was weak, relative to other inhibitors. Of importance to the current study, this weak inhibitory effect of DOX (IC_{50} of between 0.3 and 1 mM) was independent of the phorbol ester-binding site (30). Interestingly, despite the earlier report of PKC inhibition by AD 32 (14), in our hands, it proved impossible to observe either PKC-modulatory activity or rapid apoptotic cell death with this agent at concentrations $\leq$30 μM. The reasons for this disparity may relate to the assay systems involved.

The competitive inhibition of PKC by AD 198 with respect to ligands for the phorbol ester binding site, and the ability of AD 198 to compete with [^{3}H]PDBu for binding, led to the hypothesis that AD 198 interacts specifically with the phorbol binding C1-domain. Consistent with this hypothesis, in cell-free studies, kinase inhibition by AD 198 is specific for the phorbol responsive classical and novel isoforms of PKC. Furthermore, the ability of AD 198 to compete with [^{3}H]PDBu for binding to the isolated C1b domain of PKC-δ and the nonkinase phorbol ester receptor, β2-chimaerin, confirms that AD 198 interacts with C1-domains. Likewise, several pieces of evidence argue strongly against a simple membrane detergent effect by 14-*O*-acyl drugs: (*a*) AD 198 and AD 288 have been shown to partition into model membranes to a comparable extent (37), but, although AD 198 inhibits PKC activity in cell-free systems, AD 288 does not; (*b*) given the comparable lipid requirement of PKC-ζ and cPKCs, a nonspecific detergent effect by a test agent would be expected to inhibit the activity of PKC-ζ, but no such effect is evident; (*c*) as described in Ref. 38, the capacity of AD 198 to produce rapid apoptotic cell death correlates with PKC-δ activity and is modulated by the PKC inhibitor rottlerin; and (*d*) as shown here, the correlative effects of PKC modulation and cytotoxicity among a homologous series of AD 198 analogues of equal lipophilic character clearly indicate a molecular basis for the observed AD 198-PKC interaction. Thus, PKC appears to be a very significant membrane target rather than a bystander to a nonspecific membrane drug effect.

Phorbol esters bind within a groove formed between residues 6–13 and 21–27 of the 50 amino acid C1-domain consensus sequence (39). The explicit structural information available detailing ligand interactions with C1-domains has allowed us to develop a model for the binding of AD 198 to the C1b domain of PKC-δ using molecular modeling (16). In these studies, we reported that AD 198 binds to the C1b domain in a manner similar to that reported for phorbol-13-acetate in the crystal complex (39). Interestingly, this modeling study suggests that the *N*-benzyl and 14-valerate substituents of AD 198 play a similar role to the acyl chains of phorbol esters in binding to C1-domains. Specifically, these pendant moieties appear to contribute to membrane anchoring rather than direct interactions with the protein. The structure activity relationship predicted from these modeling studies is that AD 198 should be more active than either DOX or AD 288, and, as shown in the present study, this is indeed

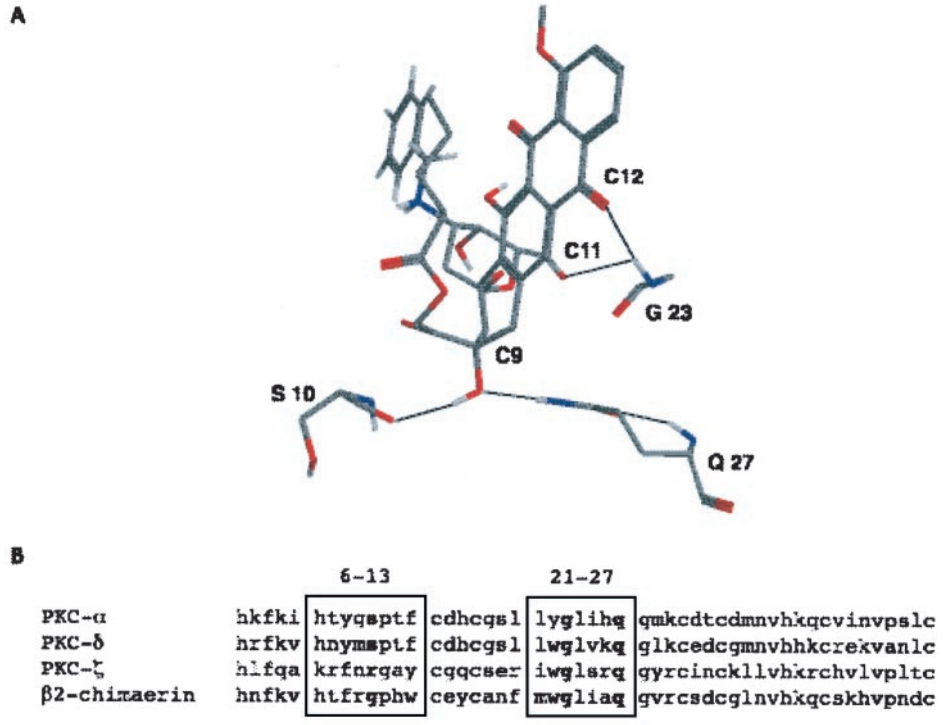

Fig. 10. *A* and *B,* proposed interactions of AD 198 with PKC-δ C1b domain and primary sequences of C1-domains *A,* hydrogen bonds formed between AD 198 and the δC1b. *B,* primary sequences of C1-domains used in the present study.

observed experimentally. A striking feature of this model is the interaction of AD 198 with residues of the δC1b that are highly conserved among other C1-domains (Fig. 10). Thus, it is not surprising that AD 198 has similar interactions with distinct phorbol ester receptors, namely cPKCs, novel PKC isoforms, and β2-chimaerin.

Our model of AD 198 bound to the PKC-δ C1b, and current models for the interaction of C1-domains with ligands and lipid bilayers allow us to propose a mechanism by which AD 198 inhibits PKC. On diacyl glycerol or phorbol ester binding to the C1-domain of PKC, the hydrophilic groove between residues 6–13 and 21–27 of the domain is capped, creating a contiguous hydrophobic surface (39). This increase in hydrophobic surface area is thought to promote insertion of the C1-domain several angstroms into the lipid bilayer (40), with concomitant activation of PKC (31). A cationic C1-ligand, such as AD 198, may occupy the C1-domain but, because of its charge, prevent appropriate insertion and activation. Consistent with this hypothesis, in our previous study (16), we reported that AD 198 promoted the membrane association of PKC-α and –δ in NIH/3T3 cells, whereas in the present study, over a similar concentration range, AD 198 blocks the activation of PKC by phorbol esters in NIH/3T3 cells. It is interesting to note that sphingosine, also a cationic PKC inhibitor with properties similar to those reported here for AD 198 (41), inhibits [^{3}H]PDBu binding to C1-domains (42). However, in contrast to AD 198, sphingosine prevents the insertion of PKC into phospholipid bilayers (25). Additional biochemical studies with site-directed mutants of the PKC-δ C1b domain (21) are currently underway to further test this model of AD 198-δC1b complex formation and this proposed mechanism of inhibition.

We recognize the potential limitations to the use of the artificial substrate, PKC-α pseudosubstrate peptide, in both the present *in vitro* and cellular studies. However, identical results were observed with other substrates, including histone and peptide substrates derived from myelin basic protein, neurogranin, and the epidermal growth factor receptor (data not shown). Nevertheless, the importance of our current observations would be strengthened by demonstration of a blockade of endogenous substrate phosphorylation and signaling pathways distal to PKC activation; such studies are currently underway in our laboratories. Interestingly, initial findings from these studies suggest that, in contrast to the inhibitory effects reported here, in some cell lines, PKC activation may occur after exposure to AD 198. In this regard, in both 32D.3 cells and squamous cell carcinomas (UM-SCC-5) of the head and neck, catalytic inhibitors of PKC attenuate the cytotoxic effects of AD 198 (43, 44). Thus, it may be that AD 198 is in fact a partial agonist of C1-domains, eliciting disparate effects in a cell type-dependent manner. In support of the hypothesis regarding a partial agonist effect for AD 198 is our recent observation that AD 198 stimulates the autophosphorylation of rat brain PKC and recombinant PKC-δ *in vitro*. The attenuation of AD 198 cytotoxicity by PKC inhibitors, such as rottlerin, are described more completely in Ref. 38.

With regard to the potential therapeutic implications of drug-PKC binding, it is interesting to note the close parallels between the CEM cytotoxicity data and the inhibition of PKC by AD 198 and the 14-acyl drug congeners. In both experimental systems, the optimal effect is observed with the valerate substituents. In the context of the present PKC studies, there are three significant points to be made concerning the

biological data: (*a*) with regard to the rapid induction of apoptosis in CEM cells, both DOX (data not shown) and AD 288, the principal biotransformation product in common for all of the 14-*O*-acyl congeners, are inactive. This ineffectiveness parallels their lack of inhibitory activity against PKC (Figs. 3*B,* 5, and 8*A*). However, it does not mean to imply that in more extended assays, *e.g.,* 48 h, these agents may not possess biological activity through "traditional" effects on DNA topo II as a result of biotransformation. The short-term conditions used here were chosen to highlight the biological differences between these congeners with respect to PKC *versus* classical DOX effects that take longer to manifest; and (*b*) although each of the congeners predictably undergoes ester hydrolysis, the rate at which this process occurs is relatively slow. Thus, ~90% of the applied concentration of congener (1–20 μM) is intact at 1 h, and ~60% remains intact after 8 h. Given that apoptotic cell death is evident in CEM cells within 3–4 h, there is therefore ample time for the intact drug congeners to have exerted a biological effect before significant biotransformation had occurred. Moreover, *N*-benzyladriamycin-14-pivalate, a hydrolysis-resistant analogue of AD 198 (18), displays equivalent activity both in terms of cytotoxicity and inhibition of [^{3}H]PDBu binding in CEM cells (Table 1). On the basis of these results, it is highly unlikely that the effect of AD 198 under these assay conditions can be attributable to AD 288, the biotransformation product.

Finally, relative to the intracellular drug concentrations achieved in these cytotoxicity assays, HPLC analysis reveals that an applied extracellular concentration of 7.5 μM AD 198 results in ~5 μmol/mg cellular protein. Significantly, separate studies show that >96% of [^{14}C]-AD 198 added to cultured cells is found within the cellular membrane fractions (45). Thus, the concentrations of AD 198 and congeners used in the *in vitro* assays are well within those achievable in cells at cytotoxic drug concentrations.

With regard to the present correlation between the structure and biochemical or biological activity of the congeners, more recent studies have indeed served to reinforce this relationship. Thus, in murine myeloid 32D cells transfected with Bcl-2 (see Ref. 38), the ability of the drug analogues to circumvent overexpression of this antiapoptotic protein correlate with the nature of the 14-acyl substituents. Once again, optimal activity is found with the valerate substituents. Bcl-2-overexpressing cells remain entirely sensitive to the effects of AD 198, whereas they exhibit resistance to AD 288 and DOX. On the basis of these two separate cellular studies, it therefore appears that the unusual biological activity of 14-acyl compounds may indeed involve a direct drug-PKC interaction. Whether the cellular effects of the 14-*O*-acyl congeners result in phosphorylation of Bcl-2, or indeed whether apoptosis occurs independently of Bcl-2, is presently under investigation. Regardless, given the significant role for Bcl-2 in the failure of many current chemotherapeutics, *e.g.,* in the treatment of prostate cancer (46), the observations concerning circumvention of this antiapoptotic protein represent a promising experimental lead.

In conclusion, the present report clearly demonstrates an interaction of AD 198 and other 14-*O*-acyl anthracycline congeners with PKC and the nonkinase phorbol ester receptor, β2-chimaerin. Although the role and precise nature of phorbol ester receptor interactions in the biological activity of these novel drugs remain to be defined, the observations presented here provide insight into the potential development of new types of resistance-bypassing drugs for use in cancer chemotherapy.

Acknowledgments

We thank Dr. Patrick J. Rodrigues for his skillful preparation of AD 198 and the other anthracyclines used in this study. We also thank Drs. Lauren Cagen, Helen Poppleton, and Tarun Patel for their thoughtful discussions and suggestions. We thank Robb Roaten for his assistance in preparing figures for this manuscript.

References

1. Riggs, C. E. Antitumor antibiotics and related compounds. *In:* M. C. Perry (ed.), The Chemotherapy Source Book, Ed. 2, pp. 345–386. Baltimore: Williams and Wilkins, 1996.

2. Myers, C. E., and Chabner, B. A. *In:* B. A. Chabner and J. M. Collins (eds.), Anthracyclines, in Cancer Chemotherapy: Principles and Practice, pp. 356–381. Philadelphia: Lipincott, 1990.

3. Gewirtz, D. A. A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. Biochem. Pharmacol., *57:* 727–741, 1999.

4. Sweatman, T. W., and Israel, M. Anthracyclines. *In:* B. A. Teicher (ed.), Cancer Therapeutics: Experimental and Clinical Agents, pp. 113–135. Totowa: Humana Press, Inc., 1997.

5. Medical Letter. March 26, 1999 (1049), p. 32.

6. Israel, M., Seshadri, R., Koseki, Y., and Idriss, J. M. Amelioration of adriamycin toxicity through modification of drug-DNA binding properties. Cancer Treat. Rev., *14:* 163–167, 1987.

7. Sweatman, T. W., Israel, M., Koseki, Y., Seshadri, R., and Beck, W. T. Cytotoxicity and cellular pharmacology of *N*-benzyladriamycin-14-valerate in mechanistically different multidrug-resistant human leukemia cells. J. Cell Pharmacol., *1:* 95–102, 1990.

8. Lothstein, L., Wright, H., Sweatman, T. W., and Israel, M. *N*-Benzyladriamycin-14-valerate and drug resistance: correlation of anthracycline structural modification with intracellular accumulation and distribution in multidrug resistant cells. Oncol. Res., *4:* 341–347, 1992.

9. Harstrick, A., Vahnhoefer, U., Schleucher, N., Schroeder, J., Baumgart, J., Scheulen, M. E., Wilke, H., and Seeber, S. (1995) Activity of *N*-benzyladriamycin-14-valerate (AD 198), a new anthracycline derivate, in multidrug resistant human ovarian and breast carcinoma cell lines. Anticancer Drugs, *6:* 681–685, 1995.

10. Mellor, H., and Parker, P. J. The extended protein kinase C superfamily. Biochem. J., *332:* 281–292, 1998.

11. Nishizuka, Y. Protein kinase C and lipid signaling for sustained cellular responses. FASEB J., *9:* 484–496, 1995.

12. Caponigro, F., French, R. C., and Kaye, S. B. Protein kinase C: a worth while target for anticancer drugs?. Anticancer Drugs, *8:* 26–33, 1997.

13. Hoffman, J. The potential for isozyme-selective modulation of protein kinase C. FASEB J., *11:* 646–669, 1997.

14. Chuang, L. F., Kung, H. F., Israel, M., and Chuang, R. Y. Activation of human leukemia protein kinase C by tumor promoters and its inhibition by *N*-trifluoracetyladriamycin-14-valerate. Biochem. Pharmacol., *43:* 865–872, 1992.

15. Wang, S., Zaharevitz, D. W., Sharma, R., Marquez, V. E., Lewin, N. E., Du, L., Blumberg, P. M., and Milne, G. W. A. The discovery of novel, structurally diverse protein kinase agonists through computer 3D-database pharmacophore search. Molecular modeling studies. J. Med. Chem., *37:* 4479–4489, 1994.

16. Roaten, J. B., Kazanietz, M. G., Sweatman, T. W., Lothstein, L., and Parril, A. Molecular models of *N*-benzyladriamycin-14-valerate in complex

with the C1-domain of protein kinase C-δ. J. Med. Chem., *44:* 1028–1034, 2001.

17. Israel, M., and Seshadri, R. US Patent 4, 610,977. Washington DC: US Patent Office, 1986.

18. Lothstein, L., Rodrigues, P. J., Sweatman, T. W., and Israel, M. Cytotoxicity and intracellular biotransformation of *N*-benzyladriamycin-14-valerate (AD 198) are modulated by changes in 14-*O*-Acyl chain length. Anticancer Drugs, *9:* 58–66, 1998.

19. Leventhal, P. S., and Bertics, P. J. Activation of protein kinase C by selective binding of arginine-rich polypeptides. J. Biol. Chem., *268:* 13906–13913, 1993.

20. Mosior, M., and Epand, R. M. Mechanism of activation of protein kinase C: roles of diolein and phosphatidylserine. Biochemistry, *32:* 66–75, 1993.

21. Kazanietz, M. G., Wang, S., Milne, G. W., Lewin, N. E., Liu, H. L., and Blumberg, P. M. Residues in the second cysteine-rich region of protein kinase-δ relevant to phorbol ester binding as revealed by site-directed mutagenesis. J. Biol. Chem., *270:* 21852–21859, 1995.

22. Caloca, M. J., Fernandez, N., Lewin, N. E., Ching, D., Modali, R., Blumberg, P. M., and Kazanietz, M. G. β2-Chimaerin is a high affinity receptor for the phorbol ester tumor promoters. J. Biol. Chem., *272:* 26488–26496, 1997.

23. Goodnight, J., Kazanietz, M. G., Blumberg, P. M., Mushinski, J. F., and Mischak, H. The cDNA sequence, expression pattern and protein characteristics of mouse protein kinase c-ζ. Gene, *122:* 305–311, 1992.

24. Kazanietz, M. G., Areces, L. B., Bahador, A., Mischak, H., Goodnight, J., Mushinski, J. F., and Blumberg, P. M. Characterization of ligand specificity and substrate specificity for the calcium-dependent and calcium-independent protein kinase c isozymes. Mol. Pharmacol., *44:* 298–307, 1993.

25. Kazanietz, M. G., Krausz, K. W., and Blumberg, P. M. Differential irreversible insertion of protein kinase c into phospholipid vesicles by phorbol esters and related activators. J. Biol. Chem., *267:* 20878–20886, 1992.

26. Sharkey, N. A., and Blumberg, P. M. Highly lipophilic phorbol esters as inhibitors of specific [^{3}H]phorbol-12, 13-dibutyrate binding. Cancer Res., *45:* 19–24, 1985.

27. Sweatman, T. W., Seshadri, R., and Israel, M. Pharmacology of *N*-benzyladriamycin-14-valerate in the rat. Cancer Chemother. Pharmacol., *43:* 419–426, 1999.

28. Mosior, M., and McLaughlin, S. Electrostatics and dimensionality can produce apparent cooperativity when protein kinase c and its substrates bind to acidic lipids in membranes. *In:* J. F. Kuo (ed.), Protein Kinase C. New York: Oxford University Press, 1994.

29. Carman, G. M., Deems, R. A., and Dennis, E. A. Lipid signaling enzymes and surface dilution kinetics. J. Biol. Chem., *270:* 18711–18714, 1995.

30. Nadatake, T., Jeng, A. Y., and Blumberg, P. M. Comparison of protein kinase c functional assays to clarify mechanisms of inhibitor action. Biochem. Pharmacol., *37:* 1541–1545, 1988.

31. Newton, A. C. Regulation of protein kinase C. Curr. Opin. Cell Biol., *9:* 161–167, 1997.

32. Quest, A. F., Bardes, E. S., and Bell, R. M. A phorbol ester binding domain of protein kinase c γ. Deletion analysis of the cys2 domain defines a minimal 43-amino acid peptide. J. Biol. Chem., *269:* 2961–2970, 1994.

33. Hurley, J. H., Newton, A. C., Parker, P. J., Blumberg, P. M., and Nishizuka, Y. Taxonomy and function of C1 protein kinase C homology domains. Protein Sci., *6:* 477–480, 1997.

34. Palayoor, S. T., Stein, J. M., and Hait, W. N. Inhibition of protein kinase C by antineoplastic agents: implications for drug resistance. Biochem. Biophys. Res. Commun., *148:* 718–725, 1987.

35. Wise, B. C., and Kuo, J. F. The modes of inhibition of acyl carnitines, adriamycin, and trifluorperazine of cardiac phospholipid-sensitive calcium-dependent protein kinase. Biochem. Pharmacol., *32:* 1259–1265, 1983.

36. Hannun, Y. A., Foglesong, R. J., and Bell, R. M. The adriamycin-iron (III) complex is a potent inhibitor of protein kinase C. J. Biol. Chem., *264:* 9960–9966, 1989.

37. Constantinides, P. P., Ghosaini, L., Inouchi, N., Kitamura, S., Seshadri, R., Israel, M., Sartorelli, A. C., and Sturtevant, J. M. Interaction of *N*-alkylanthracyclines with lipid bilayers: correlations between partition coefficients, lipid phase distributions and thermotropic behavior. Chem. Phys. Lipids, *51:* 105–118, 1989.

38. Barrett, C. M., Lewis, F. L., Roaten, J. B., Sweatman, T. W., Israel, M., Cleveland, J. L., and Lothstein, L. Novel extranuclear-targeted anthracyclines override the anti-apoptotic functions of Bcl-2 and target protein kinase C pathways to induce apoptosis. Mol. Cancer Ther., *1:* 469–481, 2002.

39. Zhang, G., Kazanietz, M. G., Blumberg, P. M., and Hurley, J. H. Crystal structure of the cys2 activator-binding domain of protein kinase C-δ in complex with phorbol ester. Cell, *81:* 917–924, 1995.

40. Hurley, J. H., and Grobler, J. A. Protein kinase C and phospholipase C: bilayer interactions and regulation. Curr. Opin. Struct. Biol., *7:* 557–565, 1997.

41. Hannun, Y. A., Loomis, C. R., Merrill, A. H., and Bell, R. M. Sphingosine inhibition of protein kinase C activity and of phorbol dibutyrate binding *in vitro* and in human platelets. J. Biol. Chem., *261:* 12604–12609, 1986.

42. Areces, L. B., Kazanietz, M. G., and Blumberg, P. M. Close similarity of baculovirus-expressed *n*-chimaerin and protein kinase-Cα as phorbol ester receptors. J. Biol. Chem., *269:* 19553–19558, 1994.

43. Barrett, C. M., Roaten, J. B., Sweatman, T. W., Israel, M., Cleveland, J. L., Steiner, M. S., and Lothstein, L. Drug-induced modulation of protein kinase C (PKC) correlates with circumvention of Bcl-2 mediated inhibition of apoptosis. Proc. Am. Assoc. Cancer Res., *41:* 822, 2000.

44. Koseki, Y., Sweatman, T. W., Savranskaya, L., and Israel, M. Potentiation of drug cytotoxicity *in vitro* with *N*-benzyladriamycin-14-valerate (AD 198). Proc. Am. Assoc. Cancer Res., *37:* 334, 1996.

45. Roaten, J. B. Characterization of the phorbol ester binding C1-domain of protein kinase C as molecular target for *n*-benzyladriamycin-14-valerate (AD 198). Doctoral Dissertation, University of Tennessee College of Medicine, Memphis TN, 2001.

46. Tang, D. G., and Porter, A. T. Target to apoptosis: a hopeful weapon for prostate cancer. Prostate, *32:* 284–293, 1997.