Protection from Doxorubicin-Induced Cardiomyopathy Using the Modified Anthracycline N-Benzyladriamycin-14-valerate (AD 198)

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Running Title: Protection From Doxorubicin Cardiotoxicity Using AD 198

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Text Pages: 26
Tables: 2
Figures: 9
References: 34
Words in Abstract: 249
Words in Introduction: 379
Words in Discussion: 1,547

Abbreviations:
AD 198 - N-benzyladriamycin-14-valerate
Dox - doxorubicin
LVPD - left ventricular developed pressure
EDP – end diastolic pressure
AMPK - AMP kinase
BCK - brain creatine kinase
iNOS - inducible nitric oxide synthase
PARP - poly ADP-ribose polymerase
TnI - troponin I
β-MHC – beta myosin heavy chain
PLB - phospholamban
PKC-ε - protein kinase C – epsilon
SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis
MALDI – matrix-assisted laser desorption/ionization mass spectrometry
KH - Krebs Henseleit Buffer
CK - creatine kinase
ROS - reactive oxygen species
DAG - diacylglycerol

Recommended Section Assignment: Cardiovascular
Abstract

The anthracycline doxorubicin (Dox) is an effective anti-tumor agent. However, its use is limited due to its toxicity in the heart. N-Benzyladriamycin-14-valerate (AD 198) is a modified anthracycline with anti-tumor efficacy similar to that of Dox, but with significantly less cardiotoxicity and potentially cardioprotective elements. In the present study, we investigated the possibility of in vivo protective effects of low dose AD 198 against Dox-induced cardiomyopathy. To do this, rats were divided into 4 groups: Vehicle, Dox (20 mg/kg; single injection Day 1), AD 198 (0.3 mg/kg per injection; injections on Day 1, 2 and 3), or a combination treatment of Dox plus AD 198. Seventy-two hours after beginning treatment hearts from the Dox group had decreased phosphorylation of AMP kinase and troponin I, and reduced poly ADP-ribose polymerase, β-tubulin, and serum albumin expression. Dox also increased the phosphorylation of phospholamban and expression of inducible nitric oxide synthase in hearts. Each of these Dox-induced molecular changes was attenuated in the Dox plus AD 198 group. In addition, excised hearts from rats treated with Dox had a 25% decrease in left ventricular developed pressure (LVDP) and a higher-than-normal increase in LVDP when perfused with a high extracellular Ca²⁺ solution. The Dox-induced decrease in baseline LVDP and hyper-responsiveness to [Ca²⁺] was not observed in hearts from the Dox plus AD 198 group. Thus Dox, with well established and efficient anti-tumor protocols, in combination with low levels of AD 198, to counter anthracycline cardiotoxicity, may be a promising next step in chemotherapy.
Introduction

The anthracycline doxorubicin (Dox) is a highly effective anti-neoplastic drug used against many cancers. However, the cumulative dosage of Dox in clinical situations is limited due to serious cardiotoxic side effects. For example, in phase III clinical trials the frequency of congestive heart failure escalated in a dose-dependent manner with an observed 5% frequency at a cumulative anthracycline dose of 400 mg/m² body surface area, 26% at 550 mg/m², and 48% at 700 mg/m² (Swain et al., 2003). Further, less obvious effects occur with exposure to anthracyclines. For example, chronic left ventricular dysfunction was detected in over 50% of asymptomatic patients treated with Dox (Gottdiener et al., 1981). These subclinical effects contribute to an inability of the heart to adapt to existing or subsequent cardiovascular disease (Priebe, 2000; Schultz et al., 2003). Due to this cardiotoxicity, a number of therapeutic strategies have been explored to decrease Dox-induced damage to the heart. One such strategy is the use of anthracycline congeners with reduced cardiotoxic potential (Frishman et al., 1996). One promising, modified anthracycline we have studied is N-benzyladriamycin-14-valerate, referred to as AD 198.

AD 198 was initially developed to circumvent mechanisms of multidrug resistance that impede successful treatment of cancer (Lothstein et al., 1998; Barrett et al., 2002; Bilyeu et al., 2004). Thus far it has been demonstrated AD 198 overcomes drug resistance of certain tumors, and has comparable tumor cytotoxicity to Dox both in vitro and in vivo (Sweatman and Israel, 1996). Further, AD 198 does not have the very serious cardiotoxic effect of standard anthracyclines, and even appears to protect the heart from ischemic-reperfusion injury (Hofmann et al., 2007). These non-cardiotoxic and cardioprotective traits are likely due to the ability of AD 198 to induce activation of PKC-ε (Hofmann et al., 2007). In the present study, we wished to expand on these studies and determine if co-therapy of AD 198 plus Dox protects the
heart from Dox-induced cardiotoxicity, and to identify molecular factors that may contribute to any cardioprotective effects of AD 198. The impetus for such a study was the hope that using low-dose AD 198 to counter anthracycline toxicity would prove to be of such apparent benefit that Dox plus AD 198 will become the standard of care in treatment for a wide range of cancers.

Methods

**Chemicals and Reagents.** Dox was purchased from Sigma-Aldrich (St. Louis, MO) and AD 198 hydrochloride salt was prepared according to previously described procedures (Lothstein et al., 1998). Dox was formulated in sterile saline while AD 198 was formulated in ethanol and polyethylene glycol. The chemical structure of AD 198 can be found in Hofmann et al., 2007.

Primary antibodies utilized were phosphorylated AMP kinase (P-AMPK; Cell Signaling #2535; 1:1000 dilution), AMP kinase (AMPK; Santa-Cruz #25792; 1:500 dilution), brain creatine kinase (BCK; Santa-Cruz #15160; 1:500 dilution), inducible nitric oxide synthase (iNOS; Santa-Cruz #651; 1:500 dilution), poly ADP-ribose polymerase (PARP; Cell Signaling #9542; 1:1000 dilution), phosphorylated troponin I (P-TnI; Cell Signaling #4004; 1:2000 dilution), TnI (Santa-Cruz #8118; 1:2000 dilution), β-myosin heavy chain (β-MHC; Chemicon #MAB1628; 1:1000 dilution), phosphorylated phospholamban (P-PLB; Millipore #07-052; 1:1000 dilution), PLB (Millipore #05-205; 1:1000 dilution), desmin (Sigma #D1033; 1:1000 dilution), and β-tubulin (Sigma #T7816; 1:2000 dilution).

**In Vivo Animal Models.** All animal procedures were approved by the University of Tennessee Institutional Animal Care and Use Committee, which is certified by the American Association for Accreditation of Laboratory Animal Care. Eight week old male Wistar rats were
randomly divided into four groups: Vehicle, Dox, Dox+AD 198, and AD 198. Dox was
administered at a single dose of 20 mg/kg body weight. AD 198 was given in 3 doses for a total
of 1mg/kg body weight every 24 hrs over a 72 hour period (Table 1). For AD 198 the final
ethanol and polyethylene glycol levels were at 1% and 2.4%, respectively, in a typical injection
volume of 700 µl. All injections were i.p.

**Cardiac Myocytes.** Ventricular myocytes were isolated from untreated rats using the
procedure of Leary et al (2008). Suspended cells were incubated for 20 min in the presence
and absence of 1 μM of the myristoylated PKC-ε V1-2 peptide inhibitor (ENZO Life Science).
Subsequently, vehicle, Dox (1 μM) and/or AD 198 (0.1 μM) were added to the cell suspension.
After 60 min Laemmli electrophoretic sample buffer with phosphatase inhibitor cocktail (Sigma
Chemical P2850) was added to stop any reactions and for cell storage.

**Protein Preparation and Western Blots.** For whole animal/heart studies, at sacrifice
hearts were minced and homogenized in a buffer containing 50 mM HEPES, 1mM EDTA, 2 mM
dithiothreitol, 10 nM okadaic acid and 1% protease inhibitor cocktail. Homogenates were then
incubated on ice for 30 minutes and centrifuged twice at 10,000 g for 10 min. The supernatant
was collected, and the protein concentration determined using the Bradford assay (Bio-Rad).
Equal amounts of protein, 10 µg / gel lane, were separated by SDS-PAGE and transferred to
PVDF membranes. For cardiac myocyte studies an equal amount of cells from each group
were collected and run on SDS-APGE and transferred. Membranes were incubated with primary
antibody, washed with TBS-T, and exposed to secondary peroxidase-coupled antibodies
(1:8000 dilution). Signals were detected using an enhanced chemiluminescence kit (Pierce
Chemical).

**Mass Spectrometry.** Mass spectrometry was done at the Hartwell Center for
Bioinformatics and Biotechnology at St Jude Children’s Research Hospital. In brief, following
separation on SDS-PAGE the protein of interest was reduced, alkylated, and a tryptic digest
prepared. Mass spectrometric analysis was performed using a matrix-assisted laser desorption/ionization (MALDI) in conjunction with tandem time-of-flight mass analyzers and associated software (Model 4700 Proteomics Analyzer from Applied Biosystems, Forest City CA). Protein assignments were made on the mass spectrometry and the tandem mass spectrometry spectra with Swissport used for protein identification.

**Langendorff-Perfused Heart Preparations.** At sacrifice hearts were excised and cannulated in ice cold modified Krebs-Henseleit (KH) buffer. KH buffer contained 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 11 mM glucose, 25 mM NaHCO3, and 1.3 mM CaCl2, pH 7.4. Hearts were then mounted on a Langendorff apparatus and perfused with oxygenated KH buffer at 37°C. Following left atriotomy, a cellophane balloon attached to a pressure transducer (BLPR, World Precision Instruments, Sarasota, FL) was inserted into the left ventricle and inflated to an initial end diastolic pressure (EDP) to maximize left ventricular developed pressure (LVDP). Hearts were paced with electrodes at 300 beats per minute and pacing voltage was set at two times the threshold value. LVDP and EDP were measured continuously throughout the experiment. Each heart underwent an initial 10 minute baseline equilibration (Pre-high Ca2+ period) followed by a 45 minute perfusion with KH containing 3.5 mM CaCl2 (High Ca2+ period). Following the high calcium perfusion, hearts were returned to KH containing 1.3 mM Ca2+ for the final 10 minutes of perfusion. Pre-high Ca2+ and High Ca2+ LVDP values were averaged over a 1 minute period at the end of each of their perfusion periods to provide values for data normalization.

**Statistical analysis.** Cumulative values are presented as mean ± standard error. Data were analyzed by an analysis of variance and a Student t-test. Statistical significance was assumed for a p<0.05.
Results

Protein Expression and Phosphorylation from In Vivo Animal Studies. Body and heart weights were not statistically different between the animal treatment groups at the time of sacrifice (Table 1). However, it is interesting to note the trend in decreased body weight of Dox treated compared to Vehicle treated animals. This is likely due to the loss of appetite associated with chemotherapy. A trend towards a decrease in body weight is not seen in the comparison of Dox plus AD 198 versus Vehicle treated animals. However, additional studies are needed to determine if this is significant.

Cardiac lysates from the 4 treatment groups run on SDS-PAGE consistently showed a Dox-induced reduction in a band at approximately 65kD - 70kD (data not shown). This protein was identified as serum albumin from rat, with a confidence interval of 100%, using MALDI. Serum albumin is present in our cardiac lysates as the minced hearts were only briefly rinsed of blood prior to homogenization, i.e. excised hearts did not undergo saline retrograde aortic perfusion to remove all blood. Subsequent analysis of whole blood samples from the 4 groups demonstrated albumin expression levels were significantly decreased after Dox treatment, and co-administration of DOX plus AD 198 attenuated this Dox-induced decrease in blood albumin (Figure 1).

Cardiac lysates from the 4 treatment groups were used to measure P-AMPK, AMPK, and BCK (Figure 2). These kinases have been associated with energy balance in the heart. Using the phosphosite-specific antibody against phospho-Thr\textsuperscript{172} of P-AMPK, we observed a 45% decrease in P-AMPK induced by Dox that was reversed by co-administration of AD 198. AMPK expression was not altered by any treatments. Dox also induced an approximate 3-fold increase in BCK, and the co-administration of Dox plus AD 198 further increased BCK.
expression. AD 198 administered alone did not alter phosphorylation of AMPK, nor AMPK and BCK expression.

Inducible NOS and PARP content were also examined in cardiac lysates (Figure 3). These enzymes have been associated with oxidative damage and apoptosis. Dox treatment markedly upregulated myocardial expression of iNOS, and this effect was attenuated by AD 198 co-administration. Dox also induced a decrease in PARP, which was reversed by AD 198 co-administration. AD 198 alone did not alter iNOS or PARP content in cardiac lysates.

Expression levels and phosphorylation of TnI and PLB, proteins associated with Ca\(^{2+}\) homeostasis, were measured in cardiac lysates (Figure 4). TnI and PLB expression did not change in any of the groups. However, P-TnI was reduced by Dox, and this effect was blocked and/or reversed by co-administration of AD 198. P-PLB was increased by Dox treatment, while Dox plus AD 198 administration reversed this effect.

Cytoskeletal proteins, desmin and β-tubulin, and the contractile protein β-MHC were also examined in cardiac lysates. There was no change in desmin expression with Dox (Figure 5). However, Dox induced a decrease in β-tubulin and an increase in β-MHC expression. The Dox-induced decrease in β-tubulin was attenuated by co-administration of AD 198, whereas co-administration of Dox plus AD 198 did not alter the Dox-induced increase in β-MHC.

**PKC-ε Inhibition in Cardiac Myocytes.** Isolated ventricular myocytes from untreated rats were pre-incubated in a saline solution with and without a PKC-ε peptide inhibitor, and then acutely exposed to vehicle, Dox, and/or AD 198. Expression levels of AMPK and TnI did not change in any of the groups (data not shown). In the absence of the PKC-ε inhibitor Dox induced a decrease in phosphorylation of AMPK and TnI that was blocked and/or reversed by co-administration of AD 198 (Figure 6). These observations are similar to that seen in the in
vivo studies (Figures 2 and 4). With pre-incubation with the PKC-ε inhibitor Dox continued to reduce phosphorylation of AMPK and TnI, but co-administration of AD 198 did not attenuate the Dox effects (Figure 6).

**Myocardial Function from In Vivo Animal Studies.** To determine if AD 198 can restore myocardial functional effects induced by Dox in vivo, we measured LVDP and EDP in excised and perfused hearts of rats pretreated as shown in Table 1. Specifically, ex vivo hearts were subjected to a normal extracellular [Ca^{2+}] for 10 min (Pre-High Ca^{2+} of 1.3 mM), switched to a high [Ca^{2+}] perfusion for 45 min (High-Ca^{2+} of 3.5 mM), and returned to the normal extracellular [Ca^{2+}] for 10 minutes (Post-High Ca^{2+} of 1.3 mM). A high [Ca^{2+}] challenge was imposed to better quantify Dox-induced effects on Ca^{2+} homeostasis, and uncover any effect co-administration of AD 198 had on Dox-induced changes in Ca^{2+} handling.

Prior to high-Ca^{2+} exposure LVDP was depressed in rats treated with Dox. This decrease was attenuated by co-administration of AD 198 (Figure 6A). During the period of high [Ca^{2+}] those hearts from rats pretreated with Dox demonstrated an exaggerated response to high extracellular Ca^{2+}. This effect was attenuated by Dox plus AD 198 pretreatment (Figures 7 and 8A). Following high [Ca^{2+}], LVDP continued to be lower in hearts from Dox treated rats (Figure 6A), and all hearts showed a significant and similar reduction in relative LVDP post-high [Ca^{2+}] (Figure 8B). Interestingly, EDP following post-high [Ca^{2+}] perfusion was significantly different between groups (Figure 6B).
Discussion

In the present study we found many, but not all, of the Dox-induced changes in select myocardial proteins were blocked or reversed by co-treatment with AD 198 (Table 2). AD 198 attenuation of Dox effects appear, in part, to be due to it’s ability to activate PKC-ε. In addition, we found that both the Dox-induced (1) decrease in left ventricular developed pressure and (2) exaggerated responsiveness to Ca^{2+} overload in excised hearts were not present in hearts from rats that had been co-treated with AD 198 (Figs 7-9). Thus, our studies support the idea that some of the underlying molecular and functional changes induced in hearts that have been exposed to Dox can be blocked or reversed by low dose, co-treatment with the modified anthracycline AD 198.

Albumin content was reduced in whole blood samples of animals that were Dox treated (Figure 1). This effect has been previously documented (Iliskovic et al., 1998). Hypoalbuminemia is associated with reduced survival in heart failure patients (Horwich et al., 2008). Mechanisms that may contribute to this include increased atrial fibrillation, a reduction in colloid oncotic pressure leading to pulmonary edema, and/or a reduction in the ability of albumin to be protective in an antioxidant or antiapoptotic capacity (Bohm et al., 2009; Horwich et al., 2008). We found AD 198 co-treatment reversed the Dox effect on albumin concentration. This is consistent with the idea that co-treatment with low levels of AD 198 may protect hearts which have been exposed to Dox.

Dox also reduced AMPK phosphorylation and increased BCK expression (Figure 2). This is consistent with observations by Tokarska-Schlattner et al (2006). AMPK plays a key role as an energy sensor and regulator of energy substrate utilization (recently reviewed in Wong et al., 2009). Reduced phosphorylation of AMPK reduces ATP availability (Wong et al., 2009) and contributes to the negative energy balance seen in Dox-treated hearts. It has been
hypothesized that increased expression of brain-type CK helps to maintain creatine kinase activity in the face of loss of muscle-type CK in the Dox-exposed heart (Tokarska-Schlattner et al., 2006). Thus, the Dox-induced increase in BCK expression may be a compensatory effect to maintain energy availability. We found AD 198 co-treatment prevented the Dox-induced decrease in phosphorylation of AMPK and further increased BCK expression. Both of these effects are consistent with the idea that co-treatment of Dox plus low levels of AD 198 would lead to hearts which have improved energy balance as compared to hearts from animals which receive only Dox treatment.

Dox increased iNOS expression and decreased PARP content (Figure 3). These observations are consistent with studies by Andreadou et al (2006), Liu et al (2006), and Zhu et al (2009). The Dox-induced increase in iNOS would increase NO, and increase the reaction of NO and superoxide anion that leads to the synthesis of peroxynitrate. Peroxynitrate is a potent cellular oxidant that damages the heart. Increased PARP cleavage, via caspases 3 and 7, is a marker of increased apoptosis (Gobeil et al., 2001). We found AD 198 co-treatment reduced the Dox-induced increase in iNOS and reduced PARP cleavage. Both of these effects are consistent with the idea that co-treatment of Dox plus low levels of AD 198 would protect hearts through reduced oxidative damage and a decreased rate of apoptosis as compared to hearts from animals which receive only Dox treatment.

Dox decreased phosphorylation of TnI and increased phosphorylation of PLB (Figure 4). The Dox-induced decrease in phosphorylation of TnI is consistent with the work of Maejima et al (2008). Reduced TnI phosphorylation has been shown to increase the Ca^{2+} sensitivity of tension and may slow myocardial relaxation. On the other hand, increased phosphorylation of PLB would increase the rate of Ca^{2+} uptake of the Ca^{2+}-ATPase on the sarcoplasmic reticulum. This would lead to an increase in the rate of relaxation and increased Ca^{2+} load within the sarcoplasmic reticulum. We found AD 198 co-treatment reversed the Dox effects such that phospho-TnI was increased and phospho-PLB decreased. Both of these effects are consistent
with the idea that co-treatment of Dox plus low levels of AD 198 would produce hearts that have a Ca$_{2+}$ sensitivity and Ca$_{2+}$ handling close to that of hearts which have not been exposed to Dox.

Dox had no effect on desmin expression, but reduced β-tubulin levels and increased β-MHC content (Figure 5). Previously, Dox has been shown to reduce desmin (Fisher et al., 2005) and β-tubulin (Dudnakova et al., 2003), and increase β-MHC (de Beer et al., 2009) content in the heart. The Dox-induced decrease in β-tubulin would alter microtubules that are (1) key to intracellular trafficking of proteins such as connexin 43 and the sodium channel (Smyth et al., 2010; Casini et al., 2010), and (2) have been implicated as mediators of stress/strain effects in the heart (Iribe et al., 2009). Microtubule disruption in the heart is associated with cardiac arrhythmias and altered Ca$_{2+}$ handling (Smyth et al., 2010; Casini et al., 2010; Iribe et al., 2009). Increased β-MHC is consistent with the onset of a hypertrophic phenotype being induced by Dox. We found AD 198 co-treatment reduced the Dox-induced decrease in β-tubulin, but did not block/reverse the increase in β-MHC. The restoration of β-tubulin to more control-like levels is consistent with the idea that co-treatment of Dox plus low levels of AD 198 would produce hearts with protected microtubules as compared to hearts from animals which received only Dox treatment. The failure of AD 198 to reverse the Dox-induced increase in β-MHC, at the time point studied, suggests Dox plus AD 198 hearts are not fully protected or restored from Dox-induced cardiotoxic effects.

Dox also reduces left ventricular developed pressure in hearts perfused with solutions containing a physiologic level of extracellular Ca$_{2+}$ and, these same hearts, had an exaggerated response to a perfusion solution containing high extracellular Ca$_{2+}$ (Figs 7-9). These effects were blocked and/or reversed by co-treatment of animals with AD 198. This supports a conclusion that AD 198 can attenuate Dox-induced functional changes in the heart.

Dox did not alter end diastolic pressure in hearts perfused with solutions containing a physiologic level of extracellular Ca$_{2+}$. This is in contrast to data from human patients in which
cumulative doses of Dox typically leads to an increase in end diastolic pressure. However, work by others suggests a Dox-induced increase in end diastolic pressure is a function of time such that at 4 weeks post-Dox injection there is no change while at 12 weeks post-injection there is an increase in left ventricular diastole diameter (Teraoka et al., 2000). Thus, caution should be used when comparing our results, using analysis at 3 days post-injection from a single dose of Dox in rats, to humans. Further, we should also note end diastolic pressure post-high Ca\textsuperscript{2+} was reduced in the Dox and Dox+AD 198 groups. Although the mechanism and consequences for such an effect is not clear, it appears AD 198 does not reverse this Dox-induced effect.

Multiple mechanisms and targets of Dox contribute to its cardiotoxicity. However, underlying much of the Dox-induced toxicity is its ability to increase reactive oxygen species (ROS; Minotti et al., 2004). The chemical modifications of Dox that produce AD 198 creates a domain within AD 198 that has a structural similarity to diacylglycerol (DAG), an endogenous ligand for protein kinase C (PKC; Roaten et al., 2001). We have previously shown AD 198 binds to PKC at its DAG binding site (Roaten et al., 2002) and this activates PKC-\(\varepsilon\) in cardiomyocytes (Hofmann et al., 2007). PKC-\(\varepsilon\) activation is thought to be cardioprotective through its ability to (1) prime sarcolemma K\textsubscript{ATP} channels to reduce action potential duration and, as such, reduce Ca\textsuperscript{2+} entry and Ca\textsuperscript{2+} overload, (2) open mitochondrial K\textsubscript{ATP} channels to prevent Ca\textsuperscript{2+} overload and volume overload of the mitochondria, and reduced mitochondrial ROS production, (3) inhibit mPTP opening to inhibit mitochondrial swelling, cytochrome C release, and the uncoupling of oxidative phosphorylation, (4) activate protein phosphatase 1 which decreases phospho-PLB to decrease Ca\textsuperscript{2+} content and Ca\textsuperscript{2+} cycling thus attenuating any rise in cytosolic [Ca\textsuperscript{2+}], and (5) activate transcription factors which increase anti-oxidant expression to reduce ROS overload (Bolli et al., 2007; Budas et al., 2007). In a previous study we demonstrated AD 198 protects from ischemia-reperfusion induced cardiac dysfunction, and that this benefit is not observed in PKC-\(\varepsilon\) knockout mice (Hofmann et al., 2008). In the present
study we show PKC-ε inhibition blocks the ability of AD 198 to reverse Dox-induced changes in phosphorylation of AMPK and TnI (Figure 6). These data support an AD 198–PKC epsilon dependent protective pathway of action. However other current findings, such as the AD 198-induced reversal in hypoalbuminemia (Figure 1), are hard to envision as attributable to PKC-ε activation and require additional investigation. Studies are also needed to specifically look at Dox pharmacokinetic / distribution in the heart in the presence of AD 198. Although, it is unlikely AD 198 is cardioprotective based solely on a mechanism in which AD 198 displaces Dox binding to targets given Dox cytotoxicity of leukemia cells is not reduced by low doses of AD 198 (unpublished observation). Thus, one mechanism, but not necessarily the only mechanism, of AD 198 effects is through PKC-ε activation.

Finally, consideration of the doses used in the in vivo studies should be considered. A past study demonstrated a single 3 mg/kg dose of AD 198 in rats resulted in an ~20% decrease in mean white blood cell (WBC) count, whereas a single 10 mg/kg dose of DOX produced an ~85% decrease in mean WBC count (Sweatman et al 1999). With both doses, the rat WBC count returned to normal levels. Thus in the present studies AD 198 is cardioprotective at a dose that has a limited hematotoxicity. This is therapeutically important in considering co-administration of both agents to protect from DOX-induced damage to the heart.

In summary, the present studies support the conclusion that in vivo, cardiotoxicity induced by Dox can be substantially countered, molecularly and functionally, through co-therapy with the modified anthracycline AD 198. Much, but not all, of this cardioprotection is due to the ability of AD 198 to activate PKC-ε. Given the extent and frequency of acute and chronic heart disease attributable to anti-tumor treatment with Dox, combination therapy of Dox plus the cardioprotective anthracycline AD 198 represents a promising next step in treatment for a wide range of cancers.
References


Footnotes

This work was supported by the American Heart Association [Grant 0855263E, PAH]; the National Institutes of Health Heart Lung and Blood Institute [Grant HL-74001, LL]; and by the American Lebanese Syrian Associated Charities (RRM).
Legends for Figures

**Figure 1.** Effects of doxorubicin (Dox) and AD 198 on albumin expression. Shown are representative Coomassie blue stained SDS-PAGE gels and cumulative analysis of albumin content in whole blood samples. Values are expressed relative vehicle and are the mean ± standard error for 3 rats. *Denotes significance as compared to vehicle.

**Figure 2.** Effects of doxorubicin (Dox) and AD 198 on enzymes associated with energy balance in the heart. Shown are representative western blots and cumulative data of phosphorylated AMP-activated protein kinase (P-AMPK; A), AMPK (B), and brain creatine kinase (BCK; C) in myocardial lysates. Values are expressed relative to the first treatment in each pairing, and are the mean ± standard error for 3 hearts. *Denotes significance as compared to the first treatment in each pairing.

**Figure 3.** Effects of doxorubicin (Dox) and AD 198 on enzymes associated with oxidative damage and programmed cell death in the heart. Shown are representative western blots and cumulative data of inducible nitric oxide synthase (iNOS; A) and poly ADP ribose polymerase (PARP; B) in myocardial lysates. Values are expressed relative to the first treatment in each pairing, and are the mean ± standard error for 3 hearts. *Denotes significance as compared to the first treatment in each pairing.

**Figure 4.** Effects of doxorubicin (Dox) and AD 198 on proteins associated with Ca^{2+} sensitivity and Ca^{2+} handling in the heart. Shown are representative western blots and cumulative data of
phosphorylated troponin I (P-TnI; A), TnI (B), phosphorylated phospholamban (P-PLB; C), and PLB (D) in myocardial lysates. Values are expressed relative to the first treatment in each pairing, and are the mean ± standard error for 3 hearts. *Denotes significance as compared to the first treatment in each pairing.

**Figure 5.** Effects of doxorubicin (Dox) and AD 198 on proteins associated with cytoskeletal structure and contractile function in the heart. Shown are representative western blots and cumulative date of desmin (A), β-tubulin (B), and β-myosin heavy chain (β-MHC; C) in myocardial lysates. Values are expressed relative to the first treatment in each pairing, and are the mean ± standard error for 3 hearts. *Denotes significance as compared to the first treatment in each pairing.

**Figure 6.** Effect of acute PKC-ε inhibition on the ability of AD 198 to reverse the Dox-induced decreases in phosphorylated AMPK and TnI. Isolated ventricular myocytes from untreated rats were pre-incubated in the presence and absence of a PKC-ε peptide inhibitor, and exposed to Dox and/or AD 198. Shown are representative western blots and cumulative date of phosphorylated AMPK (A) and TnI (B). Cumulative values are expressed relative to Vehicle, and are the mean ± standard error for 3 cell isolations. *Denotes significance as compared to Vehicle while #denotes significance as compared to AD 198 alone.

**Figure 7.** Left ventricular developed pressure (LVDP; mm Hg) and end diastolic pressure (EDP) in hearts from rats pretreated as indicated. Data are from hearts prior to a high [Ca^{2+}] challenge
(Pre-High Ca\(^{2+}\)) and following the high [Ca\(^{2+}\)] challenge (Post-High Ca\(^{2+}\)). Values are means ± standard error with an n of 10 per group.

**Figure 8.** Effects of doxorubicin (Dox) and AD 198 on relative left ventricular developed pressure (LVDP) in representative hearts from rats pretreated as indicated. Excised hearts were initially perfused for 10 min in a Krebs-Henseleit buffer containing 1.3 mM CaCl\(_2\) (Pre-High Ca\(^{2+}\)), and then switched to Krebs-Henseleit containing 3.5 mM CaCl\(_2\) (High-Ca\(^{2+}\)). High-Ca\(^{2+}\) perfusion of the heart continued for 45 min, and perfusion was then returned to Krebs-Henseleit containing 1.3 mM CaCl\(_2\) for the final 10 minutes (Post-High Ca\(^{2+}\)). LVDP is expressed as percent change from Pre-High Ca\(^{2+}\) LVDP baseline.

**Figure 9.** Relative left ventricular developed pressure (LVDP) in hearts from rats pretreated as indicated. Pre-High and Post-High Ca\(^{2+}\) was a Krebs-Henseleit buffer containing 1.3 mM CaCl\(_2\), while High-Ca\(^{2+}\) was Krebs-Henseleit containing 3.5 mM CaCl\(_2\). Data are expressed relative to the Pre-High Ca\(^{2+}\) LVDP (A), and at High-Ca\(^{2+}\) for 45 min LVDP (B). *Denotes significance as compared to same treatment Pre-High Ca\(^{2+}\) (A) or High-Ca\(^{2+}\) 45 min (B), and #denotes significance comparing Dox to all other groups at that time point.
Table I. *In vivo* treatment regime and general characteristics at time of sacrifice.

<table>
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<tr>
<th>Treatment</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Body Wt (g)$^a$</th>
<th>Heart Wt (g)$^a$</th>
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<td>• vehicle for AD 198</td>
<td>• vehicle for AD 198</td>
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<td>275 ± 14</td>
<td>0.76 ± 0.03</td>
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<tr>
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<td>• vehicle for AD 198</td>
<td>• vehicle for AD 198</td>
<td>sacrifice</td>
<td>244 ± 18</td>
<td>0.74 ± 0.04</td>
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<tr>
<td>AD 198</td>
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<td>• 0.33 mg AD 198/kg</td>
<td>• 0.33 mg AD 198/kg</td>
<td>sacrifice</td>
<td>287 ± 12</td>
<td>0.80 ± 0.01</td>
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<tr>
<td>Dox + AD 198</td>
<td>• 0.33 mg AD 198/kg</td>
<td>• 0.33 mg AD 198/kg</td>
<td>• 0.33 mg AD 198/kg</td>
<td>sacrifice</td>
<td>271 ± 17</td>
<td>0.74 ± 0.04</td>
</tr>
</tbody>
</table>

$^a$Body and heart weights have 11-13 animals in each group.
Table 2. Summary of effects on myocardial proteins.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Dox² Hearts</th>
<th>[Dox + AD 198]β Hearts</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-AMPK</td>
<td>decrease</td>
<td>blocked/reversed*</td>
</tr>
<tr>
<td>AMPK</td>
<td>no change</td>
<td>no change</td>
</tr>
<tr>
<td>BCK</td>
<td>increase</td>
<td>additional increase</td>
</tr>
<tr>
<td>iNOS</td>
<td>increase</td>
<td>blocked/reversed</td>
</tr>
<tr>
<td>PARP</td>
<td>decrease</td>
<td>blocked/reversed</td>
</tr>
<tr>
<td>P-TnI</td>
<td>decrease</td>
<td>blocked/reversed*</td>
</tr>
<tr>
<td>TnI</td>
<td>no change</td>
<td>no change</td>
</tr>
<tr>
<td>P-PLB</td>
<td>increase</td>
<td>blocked/reversed</td>
</tr>
<tr>
<td>PLB</td>
<td>no change</td>
<td>no change</td>
</tr>
<tr>
<td>Desmin</td>
<td>no change</td>
<td>no change</td>
</tr>
<tr>
<td>β-Tubulin</td>
<td>decrease</td>
<td>blocked/reversed</td>
</tr>
<tr>
<td>β-MHC</td>
<td>increase</td>
<td><strong>not</strong> blocked/reversed</td>
</tr>
<tr>
<td>Albumin</td>
<td>decrease</td>
<td>blocked/reversed</td>
</tr>
</tbody>
</table>

As compared to *Vehicle or βDox alone. *PKC-ε dependent mechanism implicated.
Figure 1.

![Diagram showing Albumin with Vehicle, Dox, Dox+AD198, and AD198 conditions]
Figure 2.

A. P-AMPK

B. AMPK

C. BCK
Figure 3.
Figure 4.

A. P-TnI

B. Tn-I

C. P-PLB

D. PLB
Figure 5.

A. Desmin

B. Beta Tubulin

C. Beta MHC
Figure 8.
Figure 9.

A

![Graph showing LVDP (% Relative to Pre-High Ca²⁺) for different treatments over time.]

B

![Graph showing LVDP (% Relative to High Ca²⁺ 45 mins) for High Ca²⁺ 45 mins and Post-High Ca²⁺ phases.]