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Amidization of Doxorubicin Alleviates Doxorubicin-Induced Contractile Dysfunction and Reduced Survival in Murine Cardiomyocytes

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Abstract

Doxorubicin is an effective anthracycline used for cancer therapy. However, the clinical application of doxorubicin has been largely limited by its irreversible cardiotoxicity, which is mainly induced by the primary amine group. In this study, we structurally modified doxorubicin by converting the primary amine into an acid-labile amide before assessing the acute cardiac effect of doxorubicin (pristine or modified) on cardiomyocyte contractile function. Contractile properties of murine cardiomyocytes were analyzed including peak shortening (PS), time-to-PS (TPS), time-to-90% relengthening (TR₉₀) and maximal velocity of shortening/relengthening ($\pm$ dL/dt). Cell toxicity and survival rate were evaluated using the MTT assay. The doxorubicin-free base was amidized by reacting with 3,4,5,6-tetrahydrophthalic anhydride (THPA) or 3,3,4,4-tetramethylsuccinic anhydride (TMSA) to yield doxorubicin-THPA or -TMSA. Acute exposure of pristine doxorubicin (10^{-9} – 10^{-5} M) for 30 min significantly prolonged TPS and TR₉₀ without affecting PS and $\pm$ dL/dt. Interestingly, doxorubicin-induced prolongation of TPS and TR₉₀ was significantly attenuated or abrogated by amidization of doxorubicin. Neither doxorubicin-THPA nor -TMSA affected PS and $\pm$ dL/dt. ROS and MTT assay revealed significantly reduced ROS production and cardiac cell toxicity from amidized doxorubicin compared with the pristine compound. Comparable cytotoxicity in human ovarian cancer SKOV-3 cells was observed between amidized and pristine doxorubicin compounds. These data provide evidence for the first time that structural modification of doxorubicin alleviates its cardiac toxicity.

Keywords

doxorubicin; amidization; cardiac myocytes; shortening; relengthening; ROS

INTRODUCTION

The anthracycline derivative doxorubicin (adriamycin) has been widely used to treat patients with neoplastic diseases. However, its clinical value is greatly compromised by the overt

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cardiac toxicity of doxorubicin which results in a late irreversible congestive cardiomyopathy namely doxorubicin cardiomyopathy usually refractory to common medications (Kalyanaraman et al. 2002; Taylor and Bulkley 1982). Clinical manifestations of doxorubicin-induced cardiotoxicity encompass electrocardiographic abnormalities such as Q-T prolongation and Q-T dispersion followed by the life-threatening doxorubicin cardiomyopathy (Chugun et al. 2000; Wang et al. 2001). Morphologically, doxorubicin exposure may result in acute and distinctive nuclear alterations associated with mild cytoplasmic changes in the heart (Taylor and Bulkley 1982). Up-to-date, the precise mechanism(s) of cardiac damages are largely unknown, making it somewhat difficult to enable development of therapies to prevent and/or treat doxorubicin cardiomyopathy. Several mechanisms have been postulated for the pathogenesis of this deadly cardiotoxicity including free radical accumulation, vasoactive amine release, myocyte damage induced by intracellular Ca^{2+} overload, inhibited expression of cardiomyocyte-specific genes, impairment in myocardial adrenergic signaling/regulation and cellular toxicity of doxorubicin metabolites such as doxorubicinol (Chugun et al. 2000; Mushlin and Olson 1988; Olson and Mushlin 1990; Shan et al. 2003). In particular, generation of reactive oxygen species (ROS) and subsequent cardiac DNA or membrane damage are thought to be the most significant mechanism underscoring the cardiac toxicity of doxorubicin (Myers et al. 1977; Rajagopalan et al. 1988). It has been demonstrated that the quinone moiety of doxorubicin is prone to form highly reactive semiquinone radicals by one-electron reduction. These doxorubicin semiquinone radicals may readily react with molecular oxygen to generate superoxide anion and other reactive oxygen species *en route* to ultimate cell injury (Lown et al. 1982; Minotti et al. 1999).

An ample of evidence has suggested that the primary amino group in doxorubicin plays an important role in its cardiotoxicity. The synthetic doxorubicin analog, hydroxyrubicin, where the primary amino group is replaced by a hydroxyl group, displayed reduced cardiac toxicity compared with doxorubicin, possibly due to decreased affinity of hydroxyrubicin to cardiolipin and deamination at the 3' position (Priebe et al. 1993). We thus hypothesized that amidization of the amine group into an acid-labile amide may alleviate the doxorubicin cardiotoxicity. The amidization procedure is rational since amide is hydrolyzed upon internalization of doxorubicin into lysosomes and a fully functioning doxorubicin can be regenerated. A number of studies have shown that β -carboxyamides of primary and second amines are acid labile and quickly hydrolyze to regenerate the amines at acidic pHs, although they are stable at neutral pH (Kluger et al. 1979; Lee et al. 2007; Xu et al. 2007). Thus the aim of this study was to determine the effect of amidization (by converting the primary amine into to β -carboxyamides) on doxorubicin-induced cardiomyocyte contractile dysfunction and cell toxicity. Our goal was to alleviate doxorubicin-induced cardiomyopathy so that prevention and management of cancer may be optimized.

METHODS

Amidization of doxorubicin

Doxorubicin hydrochloride salt (DOX, 100 mg) was dissolved in 10 ml of dimethylsulfoxide (DMSO) under constant stirring. Triethylamine (120 μl) was added and stirred for another hour. The solution was extracted using dichloromethane. The dichloromethane phase was collected and dried over anhydrous magnesium sulfate. The dichloromethane was removed with a rotary evaporator and the doxorubicin-free base was obtained. The doxorubicin free base was dried under high vacuum for 8 hrs at room temperature. Doxorubicin-free base solid (81 mg) was obtained and was amidized by reacting with 3,4,5,6-tetrahydropthalic anhydride (THPA) or 3,3,4,4-tetramethylsuccinic anhydride (TMSA). In brief, the doxorubicin-free base (20 mg) was dissolved in 5 ml of dried DMSO in a 25 ml flask. 3,4,5,6-Tetrahydropthalic anhydride (6.14 mg) was added and the reaction was stirred for 6 hrs at room temperature in

the dark. The product was purified by column chromatography. The column was first washed with anhydrous diethyl ether to remove unreacted anhydride. The amidized DOX was obtained by washing the column using DMSO. The solution was lyophilized to yield a solid (DOX-THPA, 12.5 mg). DOX amidized with TMSA (DOX-TMSA) was synthesized similarly (Fig. 1). The final concentration of DMSO used in cell culture was < 0.1% which did not exert any effect on cell contractility or cell survival.

Murine cardiomyocyte isolation

The experimental procedure used in this study was approved by the Animal Use and Care Committee at University of Wyoming and was in compliance with the provisions of the Declaration of Helsinki in 1995. In brief, hearts were rapidly removed from anesthetized mice and immediately mounted on a temperature-controlled (37°C) Langendorff system. After perfusion with modified Tyrode solution for 2 min, the heart was digested for 10 min with 0.9 mg/ml collagenase D (Boehringer Mannheim Biochemicals, Indianapolis, IN) in modified Tyrode solution. The modified Tyrode solution (pH 7.4) contained the following (in mM): NaCl 135, KCl 4.0, MgCl₂ 1.0, HEPES 10, NaH₂PO₄ 0.33, glucose 10, butanedione monoxime 10, and the solution was gassed with 5% CO₂-95% O₂. The digested heart was then removed from the cannula and left ventricle was cut into small pieces in the modified Tyrode solution. These pieces were gently agitated and the pellet of cells was resuspended in modified Tyrode solution and allowed to settle for 20 min at room temperature during which time extracellular Ca²⁺ was added incrementally back to 1.2 mM. Only rod-shaped myocytes with clear edges were selected for mechanical study (Dong et al. 2006).

Cell Shortening/Relengthening

Mechanical properties of adult murine cardiac myocytes were assessed by an IonOptix Myocam system (IonOptix Incorporation, Milton, MA, USA) as described previously (Dong et al. 2006). Cells were placed in a chamber mounted on the stage of an inverted microscope and superfused (at 30°C) with a buffer containing (in mM): 131 NaCl, 4 KCl, 1 CaCl₂, 1 MgCl₂, 10 glucose and 10 HEPES at pH 7.4. The cells were field stimulated at a frequency of 0.5 Hz. Cell shortening and relengthening were assessed using the following indices: peak shortening (PS) – indicative of peak ventricular contractility, time-to-90% PS (TPS) – indicative of systolic duration, time-to-90% re-lengthening (TR₉₀) – indicative of diastolic duration, maximal velocities of shortening (+ dL/dt) and relengthening (– dL/dt) – indicatives of maximal velocities of ventricular pressure rise/fall. To test the acute effect of doxorubicin on cardiac mechanical properties, cell shortening was recorded before and 5 minutes after each individual concentration of pristine or amidized doxorubicin (10⁻⁹ – 10⁻⁵ M) treatment.

Generation of intracellular reactive oxygen species (ROS)

Production of cellular ROS was evaluated by analyzing changes in fluorescence intensity resulting from oxidation of the intracellular fluoroprobe 5-(6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA). In brief, isolated cardiomyocytes following treatment with various concentrations of pristine or amidized doxorubicin (10⁻⁸ – 10⁻⁶ M) were loaded with 1 μM of the non-fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA, Molecular Probes, Eugene, OR, USA) at 37°C for 30 min. The myocytes were rinsed and the fluorescence intensity was then measured using a fluorescent micro-plate reader at an excitation wavelength of 480 nm and an emission wavelength of 530 nm (Molecular Devices, Sunnyvale, CA, USA). Untreated cells showed no fluorescence and were used to determine background fluorescence, which was subtracted from the treated samples. The final fluorescent intensity was normalized to the protein content in each myocyte group and was expressed as percent changes from the control value (0 concentration of doxorubicin) (Privratsky et al. 2003).

Cytotoxicity assay

The cytotoxicity assay was carried out using the (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) (MTT) cell proliferation kit (ATCC, Manassas, VA) according to the modified manufacturer's protocol. Both murine cardiomyocytes and human ovarian cancer SKOV-3 cells were seeded in 96-well plates at an initial density of 15,000 cells/well in 200 μ l of RPMI medium. Murine cardiomyocytes were used immediately and SKOV-3 cells were allowed to grow for 24 hrs. The original medium in each well was replaced with 100 μ l of fresh medium. The doxorubicin, DOX-TMSA or DOX-THPA was added to the medium. Treated cells were incubated at 37°C under a humidified air with 5% CO₂ for 4 hrs. MTT reagent (10 μ l) was added to each well and the cells were incubated for 2 hrs at 37 °C or until purple crystals were visible. Detergent reagent (100 μ l) was added to each well and then the plates were placed in a 37°C incubator for 2 hrs, or until all the crystals dissolved. The absorbance at 570 nm of the solution in each well was recorded using a microplate UVspectrometer (SpectraMax 384 Plus). Cell viability was calculated according to the protocol.

Data Analysis

A total of eight mice were used in this study. For each experimental series, data are presented as Mean $\pm$ SEM. Statistical significance ($p < 0.05$) for each variable was estimated by two-way analysis of variance (ANOVA). A Dunnett's test was used for *post hoc* analysis when required.

RESULTS

Acute exposure (30 min) of pristine and amidized doxorubicin (10^{-9} – 10^{-5} M) reduced resting cell length at the highest concentration tested with no effects at lower concentrations. Similar to our earlier report (Wold et al. 2005), pristine doxorubicin did not affect peak shortening (PS) amplitude and maximal velocity of shortening/relengthening ($\pm$ dL/dt). Similar response was observed for the two amidized doxorubicin (doxorubicin-THPA and doxorubicin-TMSA) (Fig. 2). Acute administration of pristine doxorubicin prolonged the duration of myocyte shortening (TPS) and relengthening (TR₉₀) at the concentrations of 10^{-7} M or higher. Interestingly, the threshold of doxorubicin-THPA-elicited prolongation of TPS and TR₉₀ was shifted to the right to 10^{-5} M while doxorubicin-TMSA failed to prolong TPS and TR₉₀ at the concentrations tested (Fig. 3). Given the known role of ROS production in doxorubicin-induced cardiomyocyte contractile dysfunction (Wold et al. 2005), ROS generation was measured in cardiomyocytes treated with or without pristine or amidized doxorubicin using DCF fluorescence. Results shown in Fig. 4 display that pristine doxorubicin (at 10^{-7} and 10^{-6} M) significantly enhanced ROS generation. Interestingly, amidized doxorubicin exhibited significantly reduced potency in ROS generation, indicating a likely role of dampened ROS production in reduced cardiomyocyte contractile aberration following amidization of doxorubicin. Our further evaluation of cell toxicity using the MTT assay revealed that amidization of doxorubicin significantly reduced cytotoxicity in cardiomyocytes in a comparable fashion with doxorubicin-TMSA being the least cytotoxic. However, the cytotoxicity of modified doxorubicin was not significantly different from that of the pristine doxorubicin in human ovarian cancer SKOV-3 cells (Fig. 5).

DISCUSSION

Our current work has provided evidence for the first time that amidization of doxorubicin significantly lessened doxorubicin-induced contractile dysfunction, ROS generation and cell survival in adult murine cardiomyocytes. The major mechanical interruptions in response to acute pristine doxorubicin exposure found in our study were shown as prolonged duration of

contraction and relaxation, both of which considered the hallmarks of cardiomyopathies originated from several kinds of cardiac morbidities such as heart failure, ischemia-reperfusion injury, hypertension and diabetes (Wold et al. 2001; Ye et al. 2003). Given comparable cytotoxicity maintains for SKOV-3 human ovarian cancer cells, our results suggested that structural modification of doxorubicin may greatly alleviate its cardiotoxicity, a major limitation in the anticancer therapy of the anthracycline agent.

Earlier study from our lab revealed that doxorubicin prolongs cardiomyocyte contraction and relaxation duration (TPS and TR₉₀) via p38 MAP kinase-dependent accumulation of oxidative stress (Wold et al. 2005). Interestingly, data from our current study displayed a rightward shift in the threshold of doxorubicin-THPA-induced prolongation of TPS and TR₉₀ and abrogation of doxorubicin-TMSA-induced mechanical defects in cardiomyocytes, compared with the pristine doxorubicin. Meanwhile, direct measurement of ROS generation revealed reduced ROS generating capacity in amidized doxorubicin, supporting the notion of ROS-induced oxidative damage as a major culprit factor for doxorubicin-induced cardiomyopathy (Olson and Mushlin 1990). Recent evidence has indicated that peroxynitrite formation resulted from dysregulated nitric oxide and activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP) by oxidant-mediated DNA damage may contribute to cardiotoxicity of doxorubicin (Pacher et al. 2002; Pacher et al. 2003; Pacher et al. 2007). Our earlier evidence suggested that the doxorubicin-induced prolongation of TR₉₀ may be underscored by prolongation in intracellular Ca²⁺ transient clearance (Wold et al. 2005). Although this is beyond the scope of our current study, it is quite possible that modification of doxorubicin may impose a lesser detrimental effect on intracellular Ca²⁺ clearance. Several lines of evidence have suggested the existence of an abnormal Ca²⁺ homeostasis in cardiomyocytes, which may explain mechanical abnormalities in pristine doxorubicin-induced cardiomyopathy. Doxorubicin has been reported to prolong action potential duration, inhibit gene transcription of sarco(endo)plasmic reticulum Ca²⁺-ATPase (SERCA), reduce sarcoplasmic reticulum (SR) Ca²⁺ load and subsequently Ca²⁺-induced Ca²⁺ release in isolated cardiomyocytes (Arai et al. 2000; Wang et al. 2001). The observation that acute doxorubicin exposure in our current experimental setting failed to affect any other mechanical indices such as peak shortening and maximal velocity of shortening/relengthening may suggest a significant difference in sensitivity among various cardiac contractile components upon doxorubicin exposure. For example, proteins responsible for duration of cell shortening/relengthening (TPS and TR₉₀) such as SR Ca²⁺ release channel, myosin ATPase, sarco(endo)plasmic reticulum Ca²⁺-ATPase and Na⁺/Ca²⁺ exchanger may be more sensitive to acute doxorubicin exposure than those proteins responsible for cardiac contractility such as actin, myosin and SR Ca²⁺ load. It has been shown that late stage doxorubicin-induced cardiac toxicity is associated with reduced cardiac contractility, decreased velocity of contraction/relaxation and intracellular Ca²⁺ overload, something that do not exist following acute doxorubicin toxicity (Chugun et al. 2000). The decreased contractility of individual myocytes may be attributed to their low myosin content, which may result in decreased cardiac output following doxorubicin treatment (Jones et al. 1990). It is worth mentioning that doxorubicin-induced heart failure is not associated with β -adrenoceptor desensitization, unlike heart failure induced by pressure- or volume- overload (Jones et al. 1990).

Our data revealed significantly reduced cytotoxicity in response to amidized doxorubicin exposure in cardiomyocytes but not human ovarian cancer SKOV-3 cells. One possible explanation for the lessened cardiac cytotoxicity may be that the amidized doxorubicin does not localize in lysosomes and therefore cannot efficiently regenerate pristine doxorubicin. Unlike the pristine doxorubicin which carries a cationic charge and is easily sequestered into lysosomes (Gong et al. 2003), the amidized doxorubicin is negatively charged and thus is not efficient in lysosome sequestration. We are currently exploring to amide the amine using a polymer anhydride to ensure that the amidized doxorubicin is localized in lysosomes for

regeneration. It is possible that difference between normal cardiomyocytes and cancer cells such as pH may contribute to the discrepant cytotoxic response to amidized doxorubicin. As a limitation of our study, we failed to measure the intracellular doxorubicin concentration to validate β -carboxylic acid amide hydrolysis in an acidic environment. It is expected that regenerated doxorubicin following hydrolysis may quickly bind to DNA, making it difficult to be quantitated. Further study is warranted to explore the mechanism of action behind β -carboxylic acid amide hydrolysis in cardiomyocytes and cancer cells following the amidization procedure.

In conclusion, our study demonstrates that doxorubicin cardiomyopathy characterized by prolonged duration of cell shortening/relengthening may be effectively alleviated by amidization of doxorubicin. While the precise natures of acute doxorubicin exposure-induced mechanical defects and cardiac protection from structural modification are still not clear, future studies should focus on the mechanism of action behind doxorubicin-induced ROS-dependent or independent regulation on cardiac contractile response. These approaches should be helpful to advance our knowledge of the cellular effects and toxicological profiles of doxorubicin on heart function, in order to design better therapy against doxorubicin-induced cardiomyopathy.

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Fig. 1.
Structure of amidized doxorubicin-THPA and doxorubicin-TMSA.

Fig. 2. Effect of acute treatment of pristine and amidized doxorubicin (10^{-9} – 10^{-5} M) on resting cell length (panel B), peak cell shortening (percent of resting cell length, panel C), indicative of peak ventricular contractility, and maximal velocity of shortening/relengthening ($\pm dL/dt$, panel D), indicatives of maximal ventricular pressure rise/fall in adult murine cardiomyocytes. Panel A depicts representative cell shortening traces from control, doxorubicin and doxorubicin-TMSA (10^{-5} M) groups. Mean $\pm$ SEM, $n = 65-70$ cells at each data point, * $p < 0.05$ vs. control (0 doxorubicin).

Fig. 3.

Effect of acute treatment of pristine and amidized doxorubicin (10^{-9} – 10^{-5} M) on time-to-peak shortening (panel A) - indicative of systolic duration, and time-to-90% relengthening (panel B) - indicative of diastolic duration in adult murine cardiomyocytes. Mean $\pm$ SEM, n = 65 – 70 cells at each data point, * p < 0.05 vs. control (0 doxorubicin).

Fig. 4. Effect of acute treatment of pristine and amidized doxorubicin on ROS generation in cardiomyocytes. Mean $\pm$ SEM, $n = 5-8$ independent assays, * $p < 0.05$ vs. control (0 doxorubicin), # $p < 0.05$ vs. pristine doxorubicin at the same concentration.

Fig. 5. Effect of acute treatment of pristine and amidized doxorubicin on cell survival in cardiomyocytes (panel A) and human ovarian cancer SKOV-3 cells (panel B). Mean $\pm$ SEM, $n = 5$ independent assays using triplicates, * $p < 0.05$ vs. control (0 doxorubicin), # $p < 0.05$ vs. all other doxorubicin groups at the same concentration.