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Adverse effects of doxorubicin and its metabolic product on cardiac RyR2 and SERCA2A

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Running Title: Anthracyclines alter cardiac SR Ca^{2+} cycling

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NON-STANDARD ABBREVIATIONS.
CSQ2 – cardiac isoform of calsequestrin; calsequestrin type 2.
CNBr – cyanogen bromide
daunOL – daunorubicinol
doxOL – doxorubicinol
DTT – dithiothreitol
GSSG – glutathione disulphide NHMRC – National Health and Medical Research Council
P_o – open probability
ROS – reactive oxygen species
RyR1 – skeletal isoform of the ryanodine receptor; ryanodine receptor type 1
RyR2 – cardiac isoform of the ryanodine receptor; ryanodine receptor type 2
SERCA2A – Sarco/Endoplasmic Reticulum Ca^{2+}ATPase
SR – sarcoplasmic reticulum
TCEP – Tris (2-carboxyethyl) phosphine hydrochloride

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Abstract

The use of anthracycline chemotherapeutic drugs is restricted due to potentially fatal cardiotoxic side effects. It has been hypothesized that anthracycline metabolites have a primary role in this cardiac dysfunction, however there is scarce information on the molecular interactions of these compounds in the heart. Here we provide novel evidence that doxorubicin and its metabolite, doxorubicinol, bind to the cardiac ryanodine receptor (RyR2) and to the Sarco/Endoplasmic Reticulum Ca^{2+} ATPase (SERCA2A) and deleteriously alter their activity. Both drugs (0.01 μM – 2.5 μM) activated single RyR2 channels, and this was reversed by drug washout. Both drugs caused a secondary inhibition of RyR2 activity that was not reversed by drug washout. Pre-incubation with the reducing agent dithiothreitol (DTT, 1 mM) prevented drug-induced inhibition of channel activity. Doxorubicin and doxorubicinol reduced the abundance of thiol groups on RyR2, further indicating that oxidation reactions may be involved in the actions of the compounds. Ca^{2+} uptake into sarcoplasmic reticulum vesicles by SERCA2A was inhibited by doxorubicinol, but not doxorubicin. Unexpectedly, in the presence of DTT, doxorubicinol enhanced the rate of Ca^{2+} uptake by SERCA2A. Together the evidence provided here show that doxorubicin and doxorubicinol interact with RyR2 and SERCA2A in similar ways, but that the metabolite acts with greater efficacy than the parent compound. Both compounds modify RyR2 and SERCA2A activity by binding to the proteins and also act via thiol oxidation to disrupt SR Ca^{2+} handling. These actions would have severe consequences on cardiomyocyte function and contribute to clinical symptoms of acute anthracycline cardiotoxicity.

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Introduction

Anthracyclines such as doxorubicin and daunorubicin are amoung the most effective chemotherapeutic agents used for breast cancer, leukaemia and lymphoma treatment (Trachtenberg et al., 2011). However, the use of these drugs is restricted due to an eight-fold increase in risk of potentially fatal cardiotoxicity (Cusack et al., 1995). There is no consensus on the underlying molecular mechanism(s) of cardiotoxicity. One of the more prominent hypotheses involves oxidative stress-mediated cell damage due to reactive oxygen species (ROS) production (Simunek et al., 2009). However, use of antioxidants has had disappointing outcomes in both humans and animals, whilst a third of patients administered dexrazoxane (to prevent iron-mediated ROS production) still develop heart failure, suggesting the co-existence of non-ROS mediated actions (van Dalen et al., 2009). There is mounting evidence that anthracycline-induced dysfunction of cardiomyocyte Ca^{2+} signalling pathways contributes to the cardiotoxicity (Abramson et al., 1988, Charlier et al., 2005, Feng et al., 1999, Gilliam and St Clair, 2011, Hanna et al., 2011, Ondrias et al., 1990, Park et al., 2004, van Norren et al., 2009, Zorzato et al., 1985).

Cardiac muscle contraction depends on excitation-contraction coupling, the process linking depolarization of the sarcolemma surface membrane to the mechanical contraction of the muscle fibres. The action potential is detected by dihydropyridine receptor L-type Ca^{2+} channels in the surface and transverse tubule membrane. The resultant Ca^{2+} influx activates ligand-gated cardiac ryanodine receptor (RyR2) Ca^{2+} release channels located in the sarcoplasmic reticulum (SR) Ca^{2+} store membrane. Ca^{2+} released through RyR2 raises the cytoplasmic Ca^{2+} concentration to activate contractile proteins and cause muscle contraction (systole). Cytoplasmic Ca^{2+} is reduced and SR Ca^{2+} levels are restored during relaxation (diastole), due to the return of Ca^{2+} to the SR by the SR Ca^{2+} ATPase (SERCA2A) and Ca^{2+}

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extrusion from the cytosol via the Na^+/Ca^{2+} exchanger (NCX). Changes in the function of these Ca^{2+} handling proteins are implicated in numerous pathological conditions.

RyR2 mediated Ca^{2+} release is regulated by an array of ions, accessory proteins and protein modifications. RyR2 regulatory proteins include luminal anchoring proteins triadin and junctin and the SR Ca^{2+} buffering protein calsequestrin (CSQ2), in addition to the dihydropyridine receptor and the FK506 binding proteins in the cytoplasm. Normal RyR2 gating during diastole and systole is maintained by other factors including cytoplasmic and luminal [Ca^{2+}] and post-translational modifications of RyR2, including serine phosphorylation and oxidation of cysteine thiol residues. Control of SR luminal Ca^{2+} release through the RyR2 is an important factor in maintaining Ca^{2+} homeostasis and healthy cardiac function, as disturbed RyR2 activity and abnormal SR Ca^{2+} release are now implicated in several forms of arrhythmogenesis, cardiomyopathy and heart failure (reviewed in (Kushnir and Marks, 2010)).

Both RyR2 and CSQ2 are known binding targets of daunorubicin and doxorubicin (Arai et al., 1998, Boucek et al., 1999, Charlier et al., 2005, Cusack et al., 1995, Feng et al., 1999, Gambliel et al., 2002, Park et al., 2004), with the drugs causing changes in cardiac Ca^{2+} handling, including decreased SR Ca^{2+} content and increased cytoplasmic [Ca^{2+}] during diastole (Sag et al., 2011, Wang et al., 2001). *In vitro* experiments show that anthracyclines can stimulate and inhibit RyR2 Ca^{2+} release in both cardiac and skeletal muscle (Abramson et al., 1988, Olson et al., 2000, Pessah et al., 1990), reduce the Ca^{2+} binding capacity of CSQ2 (Kang et al., 2010) and are believed to compromise SERCA2A function (van Norren et al., 2009) in skeletal muscle. In addition to binding directly to RyR2 and CSQ2, anthracyclines are also thought to decrease the number of reactive thiol groups on RyR2 either directly, or indirectly via increased ROS production (Abramson et al., 1988, Hanna et al., 2011). The

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effects of anthracyclines are compounded by the formation of the secondary alcohol derivatives, doxorubicinol (doxOL) and daunorubicinol (daunOL) when doxorubicin and daunorubicin undergo a two electron reduction of their carbonyl side chain. DaunOL was found to be more potent at modulating RyR2 release from SR vesicles (Olson et al., 2000). Both doxOL and daunOL were highly effective in inhibiting SR Ca^{2+} uptake (Cusack et al., 1993, Mushlin et al., 1993). Although doxOL and daunOL have a longer half-life and are more potent at depressing cardiac function then their parent compounds (Mushlin et al., 1993) there have only been a limited number of studies exploring the interaction between the metabolites and specific cardiac targets (see above).

In this paper, we have described the effect of a range of clinically relevant doxorubicin and doxOL concentrations on RyR2 and SERCA2A and tested our hypothesis that, as with daunorubicin, the functional effects of doxorubicin and its metabolite can be attributed to more than one mechanism. The functional effects of doxorubicin and doxOL on RyR2 single channel function and on SERCA2A Ca^{2+} handling using a spectrophotometric measure of Ca^{2+} uptake were examined We find that drugs bind to RyR2 and SERC2A and readily modify thiols on both of these proteins and as a consequence, disrupt SR Ca^{2+} signalling via multiple mechanisms.

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Materials and Methods

Materials.

Phospholipids were from Avanti Polar Lipids (Alabaster, AL, USA). SDS PAGE and Western Blot apparatus and consumables were from Bio-Rad (Gladesville, NSW, Australia). Alexa Fluor 647® C2 maleimide was from Life Technologies, (Sydney, NSW, Australia). Doxorubicinol (adriamycinol) was from Toronto Research Chemicals (Toronto, Ontario, Canada). Mouse monoclonal Anti-RyR1 (34C) is from the Developmental Studies Hybridoma Bank (Iowa City, Iowa, USA) and rabbit polyclonal anti-SERCA2A from Badrilla (Leeds, UK). All other chemicals, including doxorubicin (adriamycin) were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia).

Methods.

Single channel recording and analysis. Artificial planar bilayers separating two baths (*cis* and *trans*) were formed as described previously (Beard et al., 2002, Hanna et al., 2011). SR vesicles (50 µg) were added to the *cis* solution so that the cytoplasmic surface of the SR and RyR2 faced the *cis* solution after incorporation into the lipid bilayer. SR vesicles were incorporated in the following solutions *cis*: 230 mM CsMS, 20 mM CsCl, 1 mM $CaCl_2$, and 10 mM TES (pH 7.4); and *trans*: 30 mM CsMS, 20 mM CsCl, 1 mM $CaCl_2$, and 10 mM TES (pH 7.4). After channel incorporation, free *cis* Ca^{2+} was decreased to 1 µM with the addition of 1.32 mM BAPTA and 200 mM CsMS was added to *trans* so that [Cs^{+}] symmetrical. Free [Ca^{2+}] in all solutions was determined using a Ca^{2+} electrode. Stable control activity was recorded for 4 - 5 min before drugs were added to either the *cis* or *trans* solution. Doxorubicin/doxOL were added to the *trans* chamber, but it is unlikely that the drugs only acted only on the luminal face of the RyR2. Anthracyclines are lipid soluble, and would be

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expected to cross the bilayer with the timeframe of the experiment. To remove drugs, the *trans* chamber was perfused with drug-free solutions, which would substantially reduce the levels of drugs within both chambers.

By convention electrical potentials are expressed as cytoplasmic relative to luminal. Experimentally, the *trans* solution was held at virtual ground and voltage was applied to the *cis* solution. In each experiment single channel currents were recorded at -40 mV and +40 mV, with voltage changed every 30 s.

Data was filtered at 1 kHz and sampled at 5 kHz. The mean open times of channels varies from 1 to 10 ms, and the measured dead time for the filter(s) (using a 20% discriminator) was <200 μs and the dead time calculated for an ideal fourth-order low-pass Bessel filter (Axopatch 200A; Molecular Devices, Sunnyvale, CA) is ≤200 μs. Thus, missed events would have been few and would not have altered the average parameters (Beard et al., 2008). Single channel parameters were obtained using the Channel 2 program (developed by P. W. Gage and M. Smith, John Curtin School of Medical Research, Canberra, ACT, Australia) from 60 to 90 s of channel activity. Open probability (P_o) was measured as fractional mean current or as the fraction of time that the channel was open using threshold discriminators. Fractional mean current is the average of all data points in a recording period, divided by the maximum single channel current, and is approximately equal to the P_o measured by threshold discrimination. All experiments were performed at 23 ±2°C.

SR vesicle isolation and RyR2 purification. Cardiac SR vesicles were prepared from sheep heart (Laver et al., 1995) and RyRs were solubilised and purified from SR as described by (Dulhunty et al., 2005).

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SDS Page and Western Blot. SDS Page and Western Blot were performed according to (Laemmli, 1970, Towbin et al., 1979). Briefly, proteins were separated on 4-20 % SDS polyacrylamide gels and proteins either stained with silver stain (according to manufacturer's instructions) or transferred to PVDF membrane for western blot. PVDF was exposed to primary antibodies to RyR2 or SERCA2A and then secondary HRP-conjugated antibody, prior to chemiluminesence detection. Images were developed using a Kodak Xomat M20 film processor onto Kodak Hyper ECL film.

Determination of thiol content. SR vesicles were treated with anthracyclines and known redox agents for 30 min at room temperature. Samples were then incubated with rotation, with a 20 M excess of the thiol-reactive probe Alexa Fluor 647 C2 maleimide (which binds to accessible thiols) in the dark for 2 h. After washing samples with 10 volumes of PBS, vesicles were sedimented by centrifugation at 9500 x g for 50 - 60 min in a 3 kDa Amicon concentrator. Proteins were heated at 60°C for 10 min in non-reducing sample buffer (187.5 mM Tris-HCl, 15% glycerol and bromophenol blue (pH 6.8)) and separated by SDS PAGE. Gels were stained with Sypro Orange protein stain for 45 min in the dark. Total protein content was measured by Sypro Orange fluorescence at 300 nM and thiol content was measured by Alexa 647 fluorescence at 645 nM. Fluorescent images were photographed on a Typhoon FLA 9000 fluoroimager.

SR Ca^{2+} uptake assay. The method used to measure Ca^{2+} uptake into SR vesicles in is shown in Figure 6a. A Cary 3 spectrophotometer was used to monitor extravesicular Ca^{2+} at 710 nm, using the Ca^{2+} indicator antipyrylazo III (Dulhunty et al., 1999, Jalilian et al., 2008). Cardiac SR vesicles (200 μg) were added to a solution containing 100 mM KH_2PO_4, 0.2 mM antipyrylazo III, 1 mM Na_2ATP, and 1 mM $MgCl_2$ and the Ca^{2+} ATPase was activated by MgATP in the solution. Temperature was controlled at 25° C and solutions were continually

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stirred throughout the experiment. The SR was loaded with 3 additions of 7.5 μM Ca^{2+}. Ruthenium red was then added to block RyR2 before a final addition of Ca^{2+} so that Ca^{2+} uptake could be specifically measured from the initial rate of Ca^{2+} uptake. Vesicles were pre-incubated for 20 min with anthracyclines (or with vehicle buffer alone for control experiments) before they were added to the cuvette. In a second series of experiments, vesicles were first treated with DTT for 5 min before being incubated with doxOL. The concentration of anthracycline and DTT in the cuvette was adjusted to the incubation concentration. Calibration curves of optical density changes with addition of 12.5 to 50 μM $CaCl_2$ to the cuvette solution showed that neither anthracyclines, DTT, nor ruthenium red affected the calibration.

Anthracycline-coupled Cyanogen Bromide (CNBr) Activated Sepharose was performed as previously described(Gambetta et al., 1983, Jayaraman et al., 1992). In brief, doxorubicin or doxOL was coupled to CNBr-activated Sepharose 4B according to the manufacturer's instructions and precleared purified RyR2 (0.5 mg/ml) was incubated with anthracycline-coupled beads overnight at 4°C with rotation. Unbound protein was removed by washing with 50 mM Tris-HCl, pH 8 and proteins bound to the anthracycline-coupled CNBr activated Sepharose 4B were eluted by boiling for 1 min in sample buffer (0.05 M Tris-HCl, pH 6.8, 2% SDS, 0.01 M EDTA, 10% glycerol, 0.6 M mercaptoethanol, 0.02% bromophenol blue). The resultant supernatant was loaded onto 4 - 20% gradient gels and separated by SDS-PAGE, subject to Western Blot and immunoprobed with anti-RyR2 or anti-SERCA2A antibodies.

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Results

Anthracycline-induced RyR2 channel activation and inhibition. We have previously shown that daunorubicin has a biphasic effect on RyR2 activity, inducing an initial channel activation followed by inhibition (Hanna et al., 2011). We have now examined the functional effects of the more commonly used anthracycline doxorubicin and its primary metabolite doxOL on native RyR2 channels incorporated into lipid bilayers. Drugs, at concentrations between 0.01 and 2.5 μM, were added to the *trans* chamber and channel activity was tracked over time (Fig. 1A, B). Addition of 0.01 μM doxorubicin caused a significant 3.11±0.78 fold increase in channel open probability ($P_{o;}$ Fig. 1E). Higher concentrations of doxorubicin also induced significant RyR2 activation, although the degree of activation was not significantly higher than that caused by 0.01 μM doxorubicin and the time to activation did not differ significantly between drug concentrations (Table 1). Addition of 0.5 μM, 1 μM and 2.5 μM induced a 3.91±0.34, 4.35±1.08 and 3.28±0.67 fold increase in activity, respectively, compared to control ($P \leq 0.05$; Fig. 1C-E). From here on, all P_o changes are expressed relative to control unless stated otherwise. The similar degree of activation suggests that the activation by doxorubicin is saturated with concentrations as low as 0.01 μM.

Channel activation with 0.01 μM and 0.5 μM doxorubicin was maintained for the lifetime of the experiment, with delayed inhibition observed in only 2 of 12 channels with 0.01 μM Ca^{2+} (see below). However, a sustained inhibition (following activation) was observed with higher concentrations of doxorubicin (Fig. 1B). The activity of channels treated with 1 μM and 2.5 μM doxorubicin was reduced to 81% and 48% of control P_o, respectively (Fig. 1D, F). On average, inhibition began 11.25±3.43 min after 2.5 μM and 12.28±1.87 min after 1 μM doxorubicin addition (Table 1), and was maintained for the lifetime of the experiment. Given that two channels treated with 0.01 μM doxorubicin demonstrated inactivation, it is likely that

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lower concentrations of doxorubicin could inhibit RyR2, but that it was not observed in the time frame of the experiment. The time course of channel activation and inhibition (Fig. 1B) is consistent with the sum of two independent exponential processes that might be expected for either covalent or equilibrium binding. In each case, binding begins after a delay which presumably reflects the time taken for the drug to access its active site. We did not perform an extensive dose response study, however the data in Fig. 1C and D are consistent with Hill functions in which the concentration for 50% activation is significantly less that 0.01 μM and the concentration for 50% inhibition is between 1.0 μM and 2.5 μM, again as expected for covalent or equilibrium binding.

DoxOL elicited a similar increase in RyR2 P_o as doxorubicin (Fig. 2B), with a 3.70±0.77 fold increase in P_o (relative to control; Fig. 2C) after addition of 0.01 μM doxOL. The greatest activation (6.41±0.92 fold ($P \leq 0.05$)) was seen with 0.5 μM doxOL, and was significantly higher than the increase with 0.01 μM doxOL. Higher doxOL concentrations did not evoke a further increase in activity, suggesting that activation is saturated with 0.5 μM doxOL. The time to activation was reduced when higher anthracycline concentrations were used, as illustrated in Table 1, with maximal activation reached after 2.36±0.8 min incubation with 2.5 μM doxOL, (compared with 7.8±1.8 min after incubation with 0.01 μM doxOL). The concentration-dependence of activation of doxorubicin (and doxOL – see below) approximated a hyperbolic function, as would be expected for a classic covalent dose response for activation. The data suggest a concentration for 50% activation by doxOL that is ~0.01 μM, i.e. a lower affinity than for doxorubicin.

DoxOL was a more potent inhibiting agent than doxorubicin, as all concentrations of doxOL tested caused significant inhibition of channel activity (Fig. 2A,B), and this was most apparent at the higher drug concentrations. In addition the concentration for 50% inhibition by

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doxOL was <0.5 μM indicating a higher affinity than for lower than for doxorubicin. There was a significantly larger inhibition by doxOL compared to doxorubicin, with P_o falling to 69% of control with 0.01 μM doxOL and 24% of control with 2.5 μM doxOL (Fig. 2D). Activity in all channels treated with 2.5 μM doxOL fell below control levels, whereas activity in 3 of 9 channels treated with 0.01 μM doxOL remained higher than control. Time to doxOL-induced inhibition was concentration-dependent, with inhibition beginning between 7.7±1.2 mins (2.5 μM nM doxOL) and 17±2.3 min (10 nM doxol) after drug addition (Table 1).

Reversibility of the effects of anthracyclines on single RyR2 channels. To dissect the mechanism underlying anthracycline-induced RyR2 activation and inhibition, two sets of experiments were performed to test reversibility. Firstly, after stable activation was observed following 0.5 μM doxorubicin addition (see Fig. 1C above), the *trans* chamber was perfused with drug-free *trans* solution and RyR2 activity was compared before and after drug washout (Fig. 3A, B). As expected, 0.5 μM doxorubicin increased P_o from 0.07±0.04 to 0.23±0.06, which then fell significantly to 0.07±0.03 (Fig. 3B) almost immediately after *trans* perfusion and washout of doxorubicin. This low P_o was then maintained for the lifetime of the experiment (up to 15 min after perfusion). Similar results were observed with 0.5 μM doxOL, where drug addition caused a 5-fold increase in P_o, which fell after washout to a level comparable with control (Fig. 3C, D). Reversal of drug-induced activation following washout is characteristic of low affinity ligand binding and is also seen with daunorubicin-induced activation (Hanna et al., 2011). As doxorubicin/doxOL CNBr-activated Sepharose assays showed that both forms of the drugs bind to RyR2 (Fig. 5A, B below), it is likely that doxorubicin and its metabolite activate RyR2 by ligand binding.

The effects of anthracycline-induced RyR2 inhibition were not reversed by washout. RyR2 were first treated with 2.5 μM doxorubicin or doxOL, then the *trans* chamber was perfused

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with *trans* solution (without drug) and RyR2 activity tracked over time to determine if the inhibition was reversed upon washout. Addition of 2.5 μM doxorubicin caused an initial activation, followed by sustained channel inhibition, with P_o decreasing 20-fold to 0.004±0.002 from the control P_o of 0.08±03 (Fig. 4A, B). Perfusion of the *trans* chamber failed to reverse channel inhibition, with P_o remaining at 0.007±0.003 for 10 min after perfusion. Similarly, perfusion did not reverse doxOL inhibition (Fig. 4C, D). Indeed P_o fell significantly from 0.0119±0.003 before washout to 0.002±.001 after washout. The failure of washout to the reverse drug induced inhibition indicates that activation and inhibition are mediated by different mechanisms.

The irreversible nature of the inhibitory effects could be due to the drug binding to RyR2 with high affinity. However this seems unlikely, as inhibition requires higher drug concentrations than activation. Alternatively the inhibitory effect could be due to an irreversible modification of RyR2. In the isolated environment of the bilayer this is unlikely to be due to the formation of thiol reactive forms of the drugs, due to the absence of catalysing enzymes in our isolated preparations. Rather, the inhibitory effect is most likely to be modification of reactive thiol groups on cysteines, as anthracyclines promote disulfide formation via electron exchange reactions indicating a direct interaction between the drug molecule with cysteine thiol groups (Abramson et al., 1988, Marinov et al., 2007). High concentrations of oxidants can target less accessible thiol groups and result in RyR2 (Eager et al., 1997, Eager and Dulhunty, 1999) and RyR1 (Aghdasi et al., 1997) inhibition. This is reminiscent of the inhibition of RyR2 induced by higher concentrations of daunorubicin which we have previously shown is due to the oxidation of thiol groups (Hanna et al., 2011). To determine whether doxorubicin/doxOL induced-inhibition is due to RyR2 oxidation, channels were pre-treated with the reducing agent DTT (to protect thiol groups from disulphide formation) prior to the addition of 2.5 μM

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doxOL. DTT is only weakly lipid soluble at the pH used here (7.4) and does not cross the bilayer (Hanna et al., 2011), so was added to the *cis* chamber only, as the majority of thiol residues reside on the large cytoplasmic portion of the RyR2. Addition of 1 mM DTT did not significantly alter channel activity. Subsequent addition of 2.5 μM doxOL caused a 2.9-fold activation ($P \leq 0.05$), but no secondary inhibition. Channel activity remained high for the lifetime of the experiment (up to 35 mins after doxorubicin addition; Fig. 4E). This effect of DTT in removing inhibition suggests that doxOL does inhibit the channels by modifying thiol groups.

Modification of RyR2 thiol residues by anthracyclines. RyR2 contains 89 cysteine residues per subunit, 21 of which are available for modification (Xu et al., 1998). The results of the DTT pretreatment (above), provides compelling evidence that anthracyclines alter RyR2 Ca^{2+} handling through thiol modification. We used the thiol specific Alexa Fluor 647 C2 maleimide, to confirm that doxorubicin and doxOL both modified thiol groups on RyR2. The results show that 10 μM doxorubicin and doxOL cause a ~40% reduction in the amount of free thiol groups on RyR2, and thus directly modify the thiol groups on the protein (Fig 5C, D). The data also show that RyR2 from sheep heart have a basal level of thiol modification, as incubation with the strong reducing agent TCEP (5 mM) increased free thiol abundance (Fig 5C, D). Incubation with the oxidising agent GSSG (2 mM) prior to the thiol probe assay, decreased thiol abundance (Fig. 5C, D). Millimolar GSSG is a powerful oxidizing agent and would be expected to cause near maximal oxidation of exposed thiols on RyR2 (Zable et al., 1997). This experiment supports our hypothesis that anthracyclines increase thiol modification.

SERCA 2A function is modified by anthracyclines. Anthracyclines have been reported to also compromise SERCA2A function (Cusack et al., 1993), an action that would add to effects on

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RyRs in altering Ca^{2+} signalling. To assess the effects of doxorubicin and doxOL on SERCA2A, Ca^{2+} uptake was measured in cardiac SR vesicles. The full protocol is shown in Figure 6A. SR vesicles were partially loaded with 3 additions of Ca^{2+} before RyR2-mediated release was blocked by addition of ruthenium red. One final addition of Ca^{2+} was made in the presence of ruthenium red in order to measure SERCA2A Ca^{2+} uptake in the absence of release through RyR2. There was an immediate increase rate of Ca^{2+} uptake following ruthenium red addition as the release pathway was blocked (Fig 6A, arrow). With the final Ca^{2+} addition SERCA2A uptake rate was faster in the presence of ruthenium red than prior to ruthenium red addition. This was expected since Ca^{2+} would be released through the RyR in the absence of ruthenium red, reducing the net uptake rate. This control experiment indicates that the vesicles contain intact uptake and release pathways.

Vesicles were pre-incubated with varying concentrations of doxorubicin or doxOL for 20 min. In controls for this experiment, vesicles were incubated with vehicle alone. To exclude day to day variability, control experiments were done each day and results from drug-treated vesicles compared to the average of control experiments for that day. Pre-incubation of SR vesicles with 0.01 μM or 10 μM doxorubicin did not significantly change the Ca^{2+} uptake rate (Fig. 6B). On the other hand, doxOL inhibited Ca^{2+} uptake (Fig. 6D). Pre-incubation with 0.01 and 10 μM doxOL significantly reduced Ca^{2+} uptake to 73% - 75% of the rate in SR vesicles that had been exposed to vehicle only. Pre-incubation with the lower concentration of 0.001 μM doxOL did not significantly alter Ca^{2+} uptake rate. Taken together, these results indicate that doxOL, but not doxorubicin, is an inhibitor of SERCA2A Ca^{2+} uptake function.

SERCA2A contains 26 cysteine thiol residues, but only a limited number of these are believed to be available for modification and to have a functional effect (Adachi et al., 2004). Reducing agents activate SERCA2A, while oxidizing agents inhibit pump function (Zima and

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Blatter, 2006). To determine whether doxOL might inhibits uptake by oxidizing SERCA2A thiol residues, vesicles were pre-treated with 1 mM DTT for 5 min prior to the 20 min incubation with 0.01 μM doxOL. Control vesicles were treated with 1 mM DTT and then exposed to doxOL-free buffer. Protecting thiol groups with DTT prior to incubation with doxOL not only prevented doxOL-induced inhibition, but revealed a significant increase in Ca^{2+} uptake rate of ~30% (Fig. 6F, compared to uptake in the absence of doxOL; Fig. 6D). Taken together, these data indicate that SERCA2A function may be modulated by doxOL in two ways. Firstly, an oxidation-dependent decrease in function and secondly, an increase in Ca^{2+} uptake which is independent of thiol modification.

Dissecting the effects of doxOL on SERCA2A. The results above provide strong evidence that, like RyR2, SERCA2A dysfunction is related to anthracycline induced oxidation. To confirm this hypothesis, an assay of SERCA2A thiol groups was done to confirm that anthracyclines could directly modify SERCA2A thiol residues. The thiol probe assay revealed that SERCA2A has a basal level of thiol modification, demonstrated by the finding that TCEP could increase the abundance of free thiols, while GSSG could decrease the number of free thiols. (Fig. 7A, 7B). The level of free thiols was significantly reduced after incubation with 10 μM doxorubicin or 10 μM doxOL (Fig. 7A, 7B). That doxOL also modifies free thiols on phospholamban (Karim et al., 2001), which in turn also inhibits SERCA2A function remains a possibility. We were unable to observe any Alexa Fluor 647 C2 maleimide binding to phospholamban (data not shown), possibly due to sensitivity, since phospholamban only contains 3 cysteine residues. It is possible that this assay is not sensitive enough to detect modification of only 1 to 3 residues. Regardless of whether doxOL also oxidizes phospholamban, the uptake data and free thiol assay, indicate that doxOL treatment compromises SERCA2A function via a redox modification that can be prevented by DTT.

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To determine if doxOL's stimulatory effect on SERCA2A in the presence of DTT could be caused by doxOL binding to SERCA2A, an interaction between SERCA2A and the anthracyclines was tested using doxorubicin and doxOL coupled CNBr activated sepharose beads (see methods). Both doxorubicin and doxOL bound to SERCA2A (Fig. 7C, 7D). Therefore we propose that doxorubicin/doxOL binding to SERCA2A in the presence of DTT, increases the rate of Ca^{2+} transport into the SR (Fig. 6).

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Discussion

In this manuscript we provide new insight into the mechanisms underlying anthracycline cardiotoxicity. The effects of the doxorubicin metabolite, doxorubicinol have been characterized on single RyR2 channels and on SERCA2A, and the results provide a novel understanding of the molecular effects of this compound on cardiac Ca^{2+} handling. These data are consistent with our previous work with daunorubicin and show that the parent compounds and their metabolites have comparable effects on RyR2 activity, though only the metabolite affected SERCA2A function. The work provides further evidence that anthracyclines disrupt Ca^{2+} handling and impair cardiac function, by directly targeting major Ca^{2+} handling proteins through both ligand binding and redox modification.

RyR2 activation by anthracyclines. Channel activation by anthracyclines is fully reversible upon washout, providing strong evidence that drug binding governs the activation, as suggested earlier (Hanna et al., 2011, Pessah et al., 1990). We previously demonstrated that daunorubicin activates RyR2 in the presence and absence of CSQ2 (Hanna et al., 2011), a known anthracycline protein binding target. Effects on CSQ2 were not examined here and we cannot exclude an action on CSQ2 or another RyR2-associated protein in the effects of doxorubicin and doxOL on RyR2 activity. Daunorubicin activates both purified and native RyR2 to a similar degree (unpublished data). This, and the fact that doxorubicin and daunorubicin elicit near identical regulation of RyR2 suggest that RyR2 itself is likely to be the major protein mediating the activating effect of doxorubicin reported here. Additionally, given that the drugs bind to RyR2, it is most likely that the activation is directly on RyR2, rather than on the lipid and its interaction with a transmembrane part of RyR2.

RyR2 thiol modification by anthracyclines. For the first time, we show that doxorubicin and doxOL decrease the number of unmodified thiols on RyR2. Whilst the thiol probe assay

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illustrates only the relative change in thiol abundance, we can further quantify the data to estimate the percentage of thiol groups modified by the drugs. To do this, we make the following assumptions. 1) A fixed percentage of thiols are buried within the protein structure and inaccessible for modification. 2) Treatment with TCEP reduces all accessible thiol groups. 3) Treatment with GSSG oxidises all exposed thiol groups. Given these assumptions, we estimate that doxorubicin and doxOL modify ~60 and 80% of the accessible thiols, respectively, and are relatively strong oxidizing agents.

RyR2 inhibition by anthracyclines. Anthracyclines and other quinone containing compounds can covalently modify proteins. But it is most likely that the inhibitory action of the drugs is due to RyR2 thiol modification, rather than another form of covalent modification for the following reasons. Firstly, many anthracyclines are strong alkylating agents that add a methyl group to lysine or arginine residues. However, doxorubicin and doxOL cannot form N-alkyl adducts and are incapable of alkylation (Marchini et al., 1995, Tong et al., 1979). Secondly, quinone containing molecules can react via an arylation reaction with nucleophiles including protein thiol groups to form Michael adducts (Gant et al., 1988). However, anthracyclines are poor arylators (Feng et al., 1999), likely due to their fully substituted quinone moiety (Wang et al., 2006).

It is also unlikely that doxorubicin partitions into the hydrophobic phase of the lipid bilayer whereby induction of lipid peroxidation via iron and enzyme-dependent ROS generation (Goodman and Hochstein, 1977, Winterbourn et al., 1985) could account for inhibition. This is because, firstly, enzymes and substrates required for ROS generation via redox cycling of the anthracycline complex are unlikely to be present in sufficient quantities in the isolated lipid bilayer experiments. Secondly, NEM pre-treatment prevented daunorubicin-induced RyR2 inhibition (Hanna et al., 2011). NEM covalently binds to thiol residues, indicating that

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anthracycline inhibition of RyR2 is mediated by RyR2 thiols rather than damage to the lipid bilayer (Hanna et al., 2011).

SERCA de-regulation by anthracyclines. The reduction in SERCA2A activity occurred at relatively low concentrations of doxOL, comparable to those measured in the hearts of anthracycline treated animals and humans (~0.17-0.41 μM; (Olson et al., 1988). Disulphide formation within SERCA2A may elicit reduced Ca^{2+} uptake. That blocking disulphide formation by pre-treatment with DTT induced an increase in Ca^{2+} uptake rate was an unexpected and remarkable finding. Coupled with the finding that doxOL binds to SERCA2A, we hypothesize that, as with the activation of RyR2, doxOL enhances SERCA2A function by binding to the protein. It is also possible that DTT-induced reduction of modifiable disulphide bonds exposes additional binding sites on the protein, leading to activation. Overall, the cellular consequences of doxOL's interaction with SERCA2A would be a potent decrease in SR Ca^{2+} uptake with severe consequences on SR Ca^{2+} load and subsequent RyR2 Ca^{2+} release likely (see below).

That doxOL, but not doxorubicin, caused significant SERCA2A dysfunction again illustrates the increased potency of the metabolite. Rabbit models of anthracycline cardiotoxicity show that metabolite synthesis occurs within 45 min of initial treatment, and that the metabolite has a longer half-life in the heart than the parent compound (Olson et al., 1988). The enhanced potency of the metabolites is further illustrated when over-expression of the carbonyl reductase (which catalyses the conversion of doxorubicin to doxOL), accelerates the development of cardiomyopathy (Forrest et al., 2000), while knockdown of carbonyl reductase protects against acute cardiotoxicity (Olson et al., 2003). The enhanced efficacy of doxOL compared to doxorubicin appears to arise from the modified carbonyl sidechain (Fig. 8), The influence of this sidechain on drug action is evidenced by the differential spectrum of

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chemotherapeutic activities of doxorubicin and daunorubicin, which are distinguishable only by this different functional group (Sacco et al., 2003).

Mechanism of anthracycline modification of RyR2 thiols. The electrophilic quinone moiety on anthracyclines (Fig. 8) is likely to be the functional group that interacts with thiol residues via electron transfer reactions. We cannot, however, exclude the contribution of thiol interactions with other anthracycline functional groups, including the carbonyl sidechain (see above).

The relatively negative redox potential of anthracyclines (-348mV) compared to other, less substituted quinone compounds may explain the delayed onset of thiol oxidation in our system (Butler and Hoey, 1993). It is generally accepted that most RyR thiol residues have a pKa of ~8, where they are relatively inert. However, a subset of thiols are postulated to exist within a cellular microdomain which lowers their pKa and favours the thiolate anion form of these thiols, rendering them hyperreactive (Burgoyne et al., 2012, Donoso et al., 2011, Wall et al., 2012). Whilst seven hyperreactive cysteine residues have been identified on RyR1 (Voss et al., 2004), the identity of these residues on RyR2 and whether this microdomain is conserved in cardiomyocytes, is unknown.

Synergistic effects of anthracyclines in the heart. We believe the inhibitory effects on RyR2 contribute to the acute or early phase of anthracycline cardiotoxicity, where two of the earliest symptoms are a reduced left ventricular ejection fraction and arrhythmogenesis (reviewed in (Menna et al., 2012)). Alterations in SR Ca^{2+} release and uptake play a prominent role in depressed contractility and impaired relaxation associated with anthracycline cardiotoxicity (Boucek et al., 1997, Mushlin et al., 1993, Shadle et al., 2000). This is supported by anthracycline treatment animal models where reduced Ca^{2+} transient amplitudes, increased diastolic Ca^{2+} leak and depleted SR load are observed (Sag et al., 2011, Wang et al., 2001).

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It is tempting to speculate how alterations in RyR2 and SERCA2A function by anthracyclines might lead to arrhythmia. RyR2 activation by low drug concentrations would lead to excess diastolic SR Ca^{2+} release and to delayed after-depolarisations, both well-recognized mechanisms of activity triggered in stress-induced arrhythmia (Paavola et al., 2007). Furthermore, sustained RyR2 inhibition by doxorubicin and doxOL could induce arrhythmogenic early after-depolarizations, a form of triggered activity that occurs before cell repolarization is complete (Bers, 2002). Depressed SERCA2A function is also common in pathological conditions and contributes to systolic and diastolic dysfunction in heart failure (Bers et al., 2003), where depletion of SR Ca^{2+} load contributes to Ca^{2+} release dysfunction, after-depolarization generation and reduced cardiac contractility.

What would be the consequences in the intact cell, where anthracyclines will undoubtedly induce ROS (Sag et al., 2011)? Our *in vitro* experiments were done in the absence of mitochondria and ROS producing enzymes. Therefore the effects we have characterized would (a) be additive with ROS-induced effects and (b) could constitute the cardiotoxicity pathway in patients treated with dexrazoxane to suppress ROS. Thiol modifications due to ROS (including S-glutathionylation and S-nitrosylation) also promote arrhythmogenesis and heart failure via RyR2 dysregulation (Belevych et al., 2009, Donoso et al., 2011, Yano et al., 2005). The confirmation of dual underlying mechanisms and the differential effects of doxorubicin and doxOL highlight the complexity of anthracycline cardiotoxicity. That anthracyclines could induce additional RyR2 modifications in the cell as a result of oxidative stress would lead to a complex dysfunction of RyR2 and perhaps SERCA. This compounded dysregulation is likely to be a significant cause of the cardiotoxic side effects observed in patients treated with anthracyclines.

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Authorship Contributions

Participated in research design: Hanna, Dulhunty and Beard

Conducted experiments: Hanna, Lam and Tham

Performed data analysis: Hanna, Dulhunty and Beard.

Wrote or contributed to the writing of the manuscript: Hanna, Dulhunty and Beard.

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Footnotes

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Figure Legends

Figure 1. Doxorubicin has a biphasic action on native RyR2 channels. (**A**) – (**B**). Running histogram of the experiment. RyR2 channel open probability (P_o) was measured every 10 s throughout an experiment before (white bins) and after (black bins) the addition of 0.5 μM doxorubicin (**A**) or 2.5 μM doxorubicin in (**B**) at +40 mV. (**C**) – (**D**) Three s recordings of native RyR2 channel activity at +40 mV. Channels open upward from zero current (C, continuous line) to maximum open conductance (O, dashed line). (**C**) Top panel, control recording of native RyR2 activity in the absence of doxorubicin; middle and bottom panel, after the addition of 0.5 μM doxorubicin to the *trans* chamber. Maximal RyR2 activity was first measured at 2 min in this channel (middle panel), with no further change in activity recorded for 22 min after doxorubicin addition (bottom panel). **(D)** Top panel, control recording of RyR2 activity in the absence of doxorubicin; middle and bottom panel, after the addition of 2.5 μM doxorubicin to the *trans* chamber. Maximal RyR2 activation was first seen at 2.5 min (middle panel), and maximal RyR2 inhibition was recorded 16 min after doxorubicin addition (bottom panel). (**E**) – (**F**) Combined data from measurements of P_o at +40 mV and −40 mV (n = 8 - 17). Data is presented as average P_o relative to control P_o during activation in (**E)** and during inhibition in (**F)**. Asterisk (*) indicates a significant difference from the control P_o. Crosshatch (#) indicates a significant difference between P_o during the activation and inhibitory phase.

Figure 2. DoxOL has a biphasic effect on native RyR2 channels. (**A**). Recordings of native RyR2 channel activity at -40 mV. Channels open downward from zero current (C, continuous line) to maximum open conductance (O, dashed line). Left panel, control recording of native RyR2 activity in the absence of doxOL; centre and right panel, after the addition of 2.5 μM doxOL to the *trans* chamber. Maximal RyR2 activity was measured at 1 min after addition of

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doxOL (centre panel) and maximal RyR2 inhibition measured at 4 min after doxOL addition (right panel). (**B**) Running histogram, where native RyR2 channel open probability (P_o) was measured every 10 s at +40 mV throughout an experiment before (white bins) and after (black bins) the addition of 2.5 μM doxOL. (**C**) – (**D**) Combined data from measurements of P_o at +40 mV and −40 mV (n = 7 -18 at each concentration). Data is presented as average P_o relative to control P_o during activation in (**E)** and during inhibition in (**F)**. Asterisk (*) indicates a significant difference from the control P_o recorded before adding doxOL. Crosshatch (#) indicates a significant difference between *P*o during the activation and inhibitory phase.

Figure 3. Anthracycline-induced activation is reversed by removal of the drug.
Recordings of native RyR2 channel activity at -40 mV (**A**) and +40 mV (**C**). Channels open downward **(A)** or upward **(C)** from zero current (C, continuous line) to maximum open conductance (O, broken line). Top panel, control native RyR2 recording in the absence of doxorubicin (**A**) or doxOL (**C**); middle panel, after the addition of 0.5 μM doxorubicin (**A**) and doxOL (**C**) to the *trans* chamber. Maximal activity was measured 4 (**A**) or 8 (**C**) mins after doxorubicin addition (A) and 8 min after doxOL addition (C); bottom panel, after the *trans* chamber was perfused with *trans* solution,. (**B**) and (**D**), combined data from measurements of P_o at +40 mV and -40 mV (n = 6 - 10). Average P_o before 0.5 μM doxorubicin addition, during activation and after washout of doxorubicin are shown in (**B**). Average P_o before 0.5 μM doxOL addition, during activation and after washout of doxOL are shown in (**D**). Asterisk (*) indicates a significant difference from the control P_o recorded before adding doxorubicin/doxOL. Crosshatch (#) indicates a significant difference between P_o in the presence of doxorubicin/doxOL and after drug washout.

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Figure 4. Anthracycline-induced inhibition is not reversed by removal of the drug. (**A**) and (**C**). Recordings of native RyR2 channel activity at +40 mV. Channels open upward from zero current (C, continuous line) to maximum open conductance (O, broken line). Top panel, control native RyR2 recording in the absence of doxorubicin (**A**) and doxOL (**C**); middle panel, after the addition of 2.5 μM doxorubicin (**A**) and doxOL (**C**) to the *trans* chamber minimal activity was measured 5.5 min after doxorubicin **(A)** and doxOL **(C)** addition; bottom panel, after the *trans* chamber was perfused with drug free solution. (**B**) and (**D**), Combined data from measurements of P_o at +40 mV and -40 mV (n = 8 in **B** and **D**). Average P_o before 2.5 μM doxorubicin addition, during the inhibition phase, and after washout of doxorubicin are shown in **B)**. Average P_o before 2.5 μM doxOL addition, during the inhibition phase and after washout of doxOL are shown in (**D**). **(E)** Combined average data from experiments (+40 mV and -40 mV (n = 10), where 1 mM DTT was first added to the *cis* chamber, followed by addition of 2.5 μM doxOL. In (**B**) – (**D**), Asterisk (*) indicates a significant difference from the control P_o recorded before adding dox/doxOL. In (**E**), Asterisk (*) indicates a significant difference from the P_o recorded after addition of DTT, but prior to doxOL.

Figure 5. Doxorubicin and doxOL bind to RyR2 and modify RyR2 thiols. (**A**) Western blot showing RyR2 bound to CNBr-activated Sepharose in the absence of doxorubicin/doxOL (non-specific binding; lane 1), to doxOL coupled CNBr-activated Sepharose (lane 2) and doxorubicin coupled CNBr-activated Sepharose (lane 3). (**B**) Average density of RyR2 from control (bin 1), doxOL coupled CNBr-activated Sepharose (bin 2) and doxorubicin coupled CNBr-activated Sepharose (bin 3). Asterisk (*) indicates a significant difference in densitometry units between the control and doxorubicin coupled CNBr-activated Sepharose. (**C**) SR vesicles were treated with TCEP, GSSG and anthracyclines and separated by SDS

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PAGE. Top panel - total protein was stained with Sypro Orange. Bottom panel - free thiol residues were probed with Alexa 647. (**D**) Average density units/unit RyR2, illustrating the number of thiol residues per unit of RyR2 in. Data is presented as relative to the density units/unit untreated RyR2. Asterisk (*) indicates a significant difference from non-treated (ctrl) samples.

Figure 6. SERCA2A inhibition by doxOL is prevented by DTT. (A) Experimental protocol. Ca^{2+} uptake from SR vesicles was monitored after Ca^{2+} additions using antipyrylazo III. (**B**) SERCA2A mediated Ca^{2+} uptake from the bathing solution into the SR of vesicles pre-incubated with doxorubicin **(B)** or doxOL **(D)**. The initial slope of the uptake curve was measured (dashed lines) and converted into an uptake rate. Average Ca^{2+} uptake rate of doxorubicin **(C)** and doxOL **(E)** pre-treated vesicles, relative to the uptake rate in the absence of anthracycline preincubation. (**F**) In another set of experiments vesicles were exposed to DTT for 5 min before being incubated with 0.01 μM doxOL for 20 min. The uptake rate was expressed relative to the uptake rate measured in vesicles pre-incubated with DTT alone. The average uptake rate in the presence of 0.01 μM doxOL is included for comparison. Asterisk (*) indicates a significant difference to non-treated vesicles ($p<0.05$), $n = 8 – 10$.

Figure 7. Doxorubicin and doxOL bind to SERCA2A. (**A**) SR vesicles were treated with TCEP, GSSG and anthracyclines and separated by SDS PAGE. Top panel, total protein was stained with Sypro Orange. Bottom panel, free thiol residues were probed with Alexa 647. There was no visible Alexa 647 binding to SERCA2A. (**B**) Average density units/unit SERCA2A illustrating the number of thiol residues per unit of SERCA2A protein. Data is presented as relative to the density units/unit untreated SERCA2A. Asterisk (*) indicates a significant difference from non-treated (ctrl) samples. (**C**) Western blot showing SERCA2A bound to CNBr-activated Sepharose in the absence of doxorubicin/doxOL (non-specific

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binding; lane 1), to doxOL coupled CNBr-activated Sepharose (lane 2) and to doxorubicin coupled CNBr-activated Sepharose (lane 3) (**D**) Average density of SERCA2A from control (bin 1), doxOL-coupled CNBr-activated Sepharose (bin 2) and doxorubicin-coupled CNBr-activated Sepharose (bin 3). Asterisk (*) indicates a significant difference in densitometry units between the control and doxorubicin coupled CNBr-activated Sepharose.

Figure 8. Possible sites of interaction between anthracyclines and cysteine thiol groups. The quinone moiety (solid box) of doxorubicin (CID 31703; http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=31703) and doxorubicinol (CID 8397; http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=83970&loc=ec_rcs) undergoes enzyme catalysed redox cycling and is a possible site of direct interaction with cysteine thiol residues. Another candidate for direct interaction with cysteine thiols is the carbonyl side chain (dashed boxes). Two electron reduction of the carbonyl side chain of doxorubicin yields the more potent secondary alcohol metabolite doxorubicinol. The carbonyl side chain is also an important determinant of the chemotherapeutic actions of anthracyclines, with variances here differentiating doxorubicin from daunorubicin. Chemical structures modified from images created by Pubchem compound database (National Center for Biotechnology Information).

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Table 1. **Time to effect of anthracyclines on RyR2 single channel activity**. Average time to RyR2 activation and time to RyR2 inhibition are presented for all tested concentrations of doxorubicin and doxOL. The time to activation reflects the time from when the drug was added to the chamber, to the beginning of the activation phase or inhibition phase. All times are for addition of drug to the *trans* chamber. The averages reflect the time required for the drugs to associate with their binding sites are consistent with an expected exponential time course. A significant difference in time to effect compared to previous concentrations is indicated by crosshatch (#). A significant difference in time to effect between matched concentrations of doxorubicin and doxOL is indicated by asterisk (*).

[Dox]	Time to activation (min)	Time to inhibition (min)	[Doxol]	Time to activation (min)	Time to inhibition (min)
10nM	5.2±0.7	---	**10nM**	7.8±1.8	17±2.3
500nM	4.6±0.8	---	**500nM**	4.9±1.4 (*)	14.4±1.1
1µM	4.1±1.1	11.3 ± 2.7	**1µM**	4.1±1.2	16.4±0.8
2.5µM	4.1±0.9	11.2 ± 1.3	**2.5µM**	2.3±0.8 (#) (*)	7.7±1.2 (#) (*)

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Figure 1

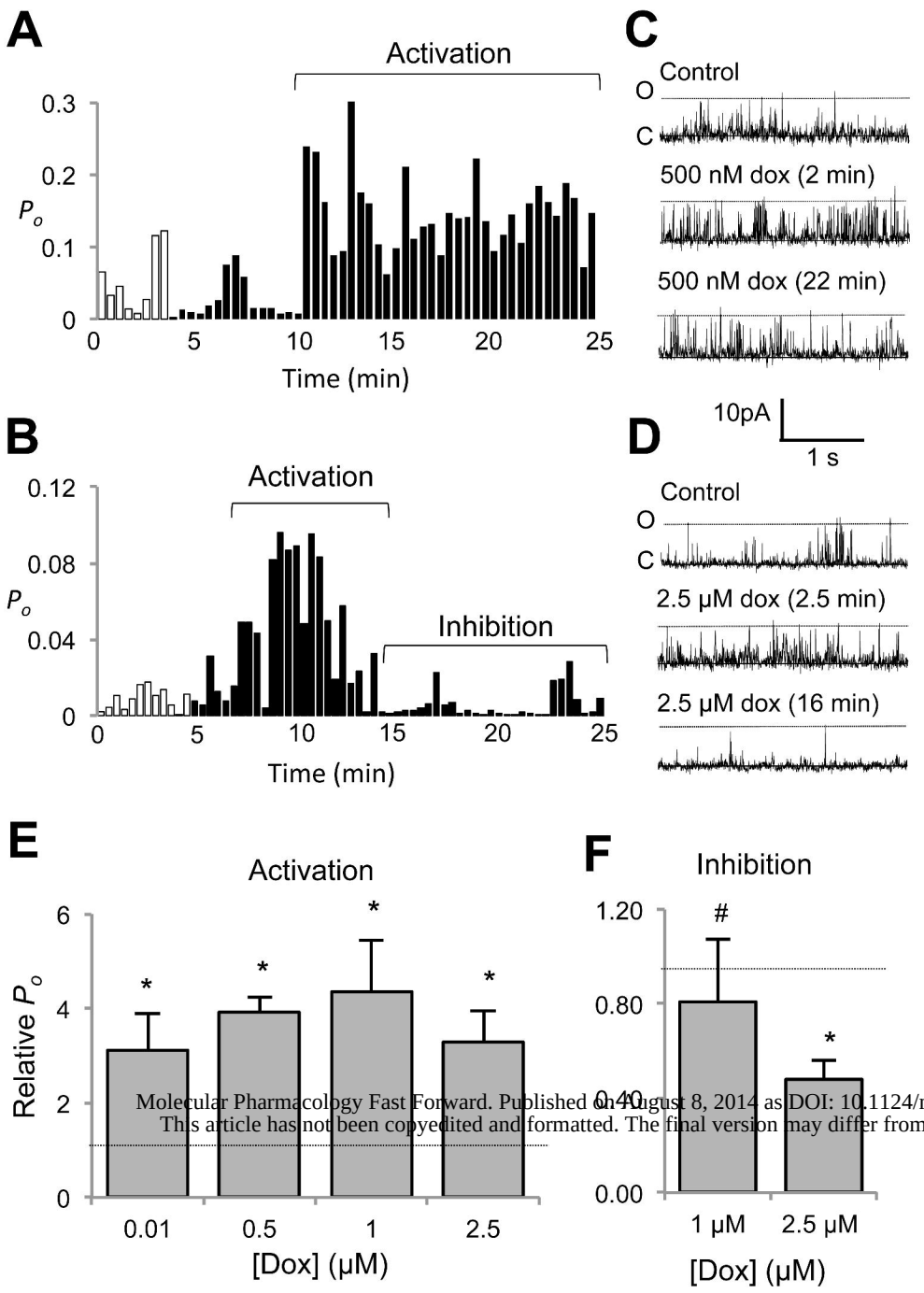

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Figure 2

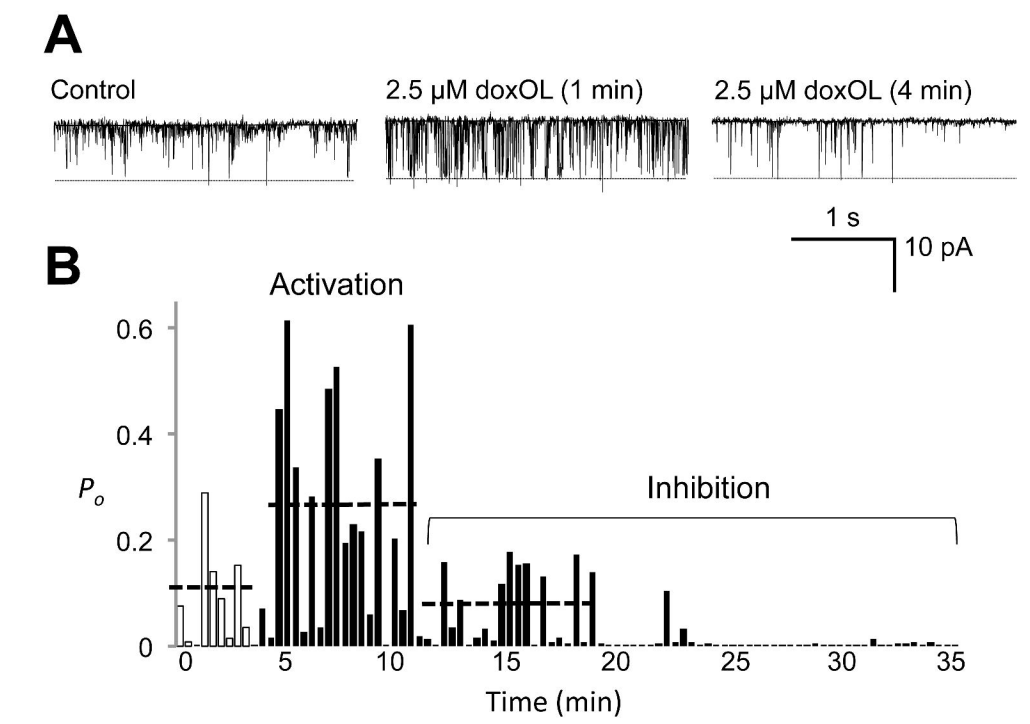

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Figure 3

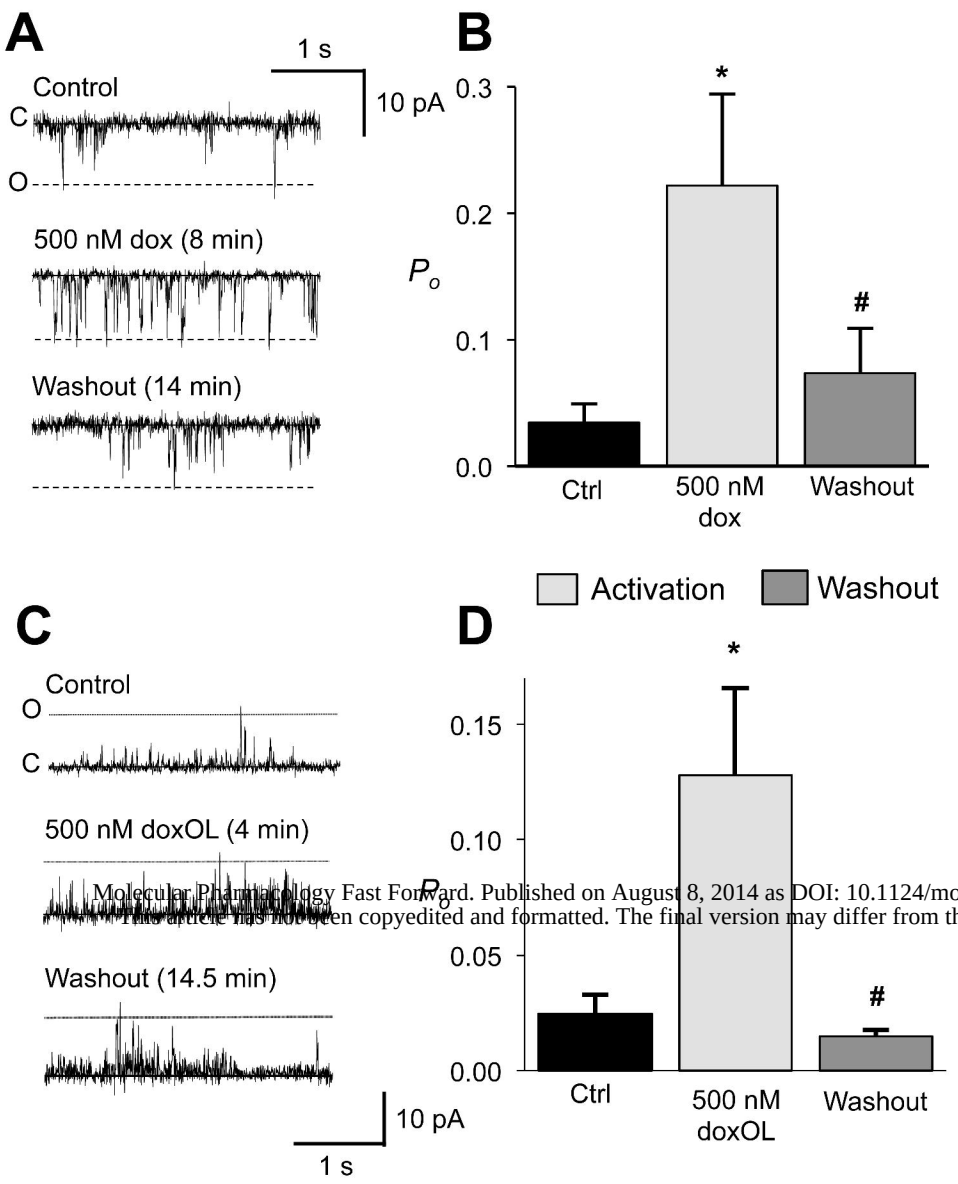

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Figure 4

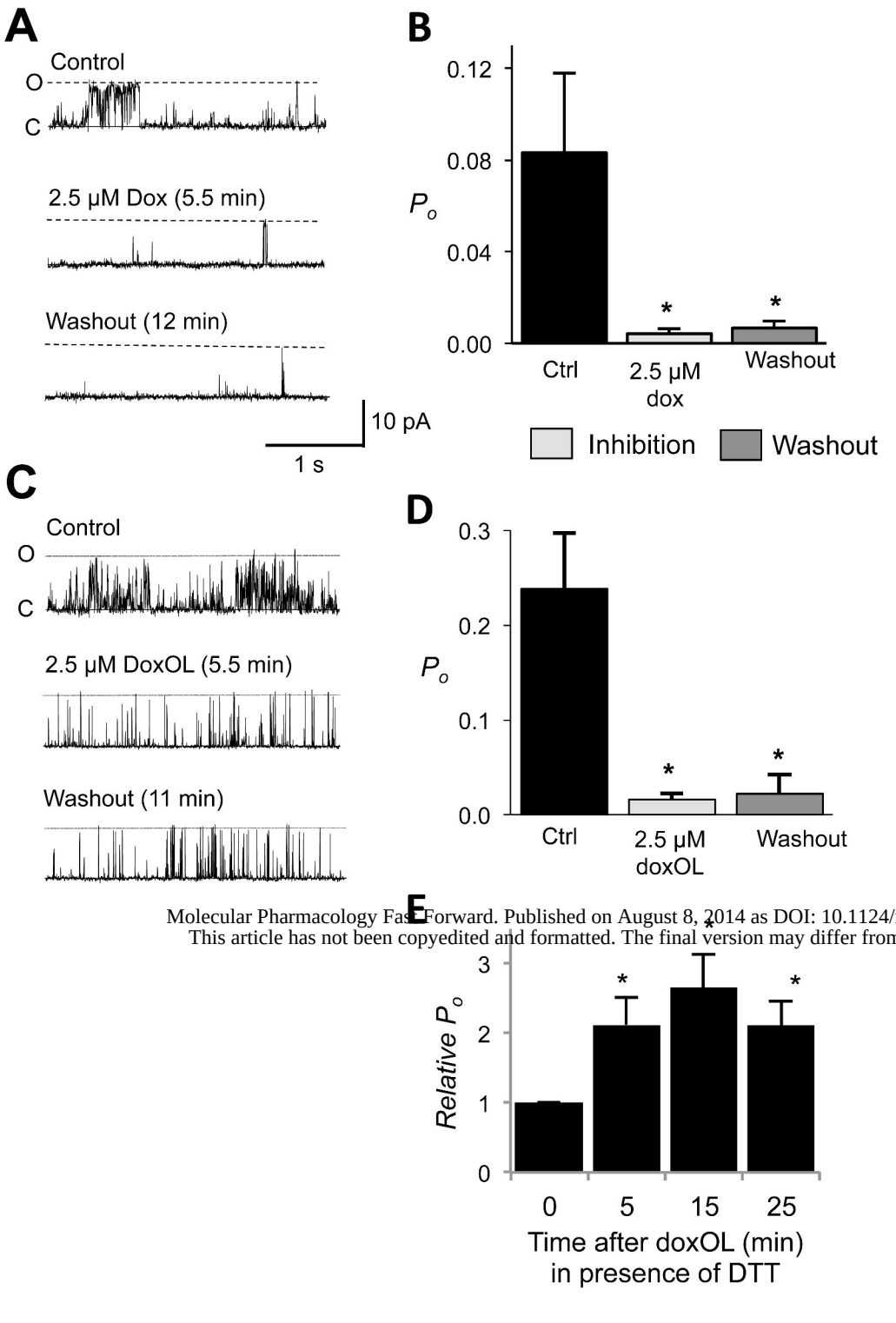

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Figure 5

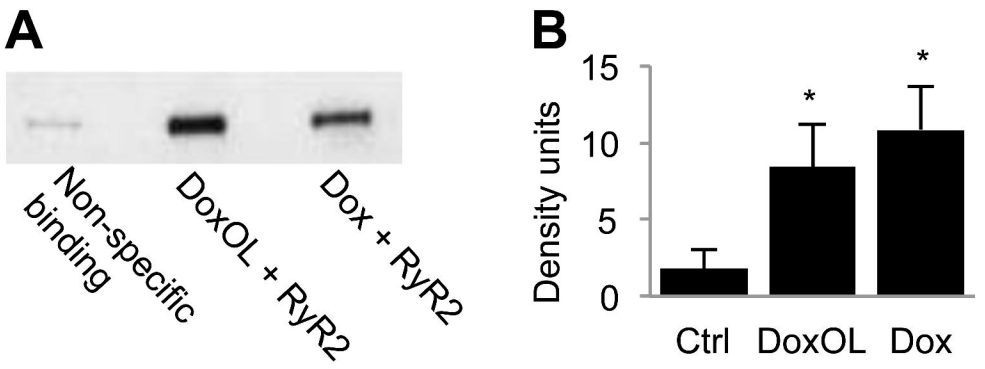

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Figure 6

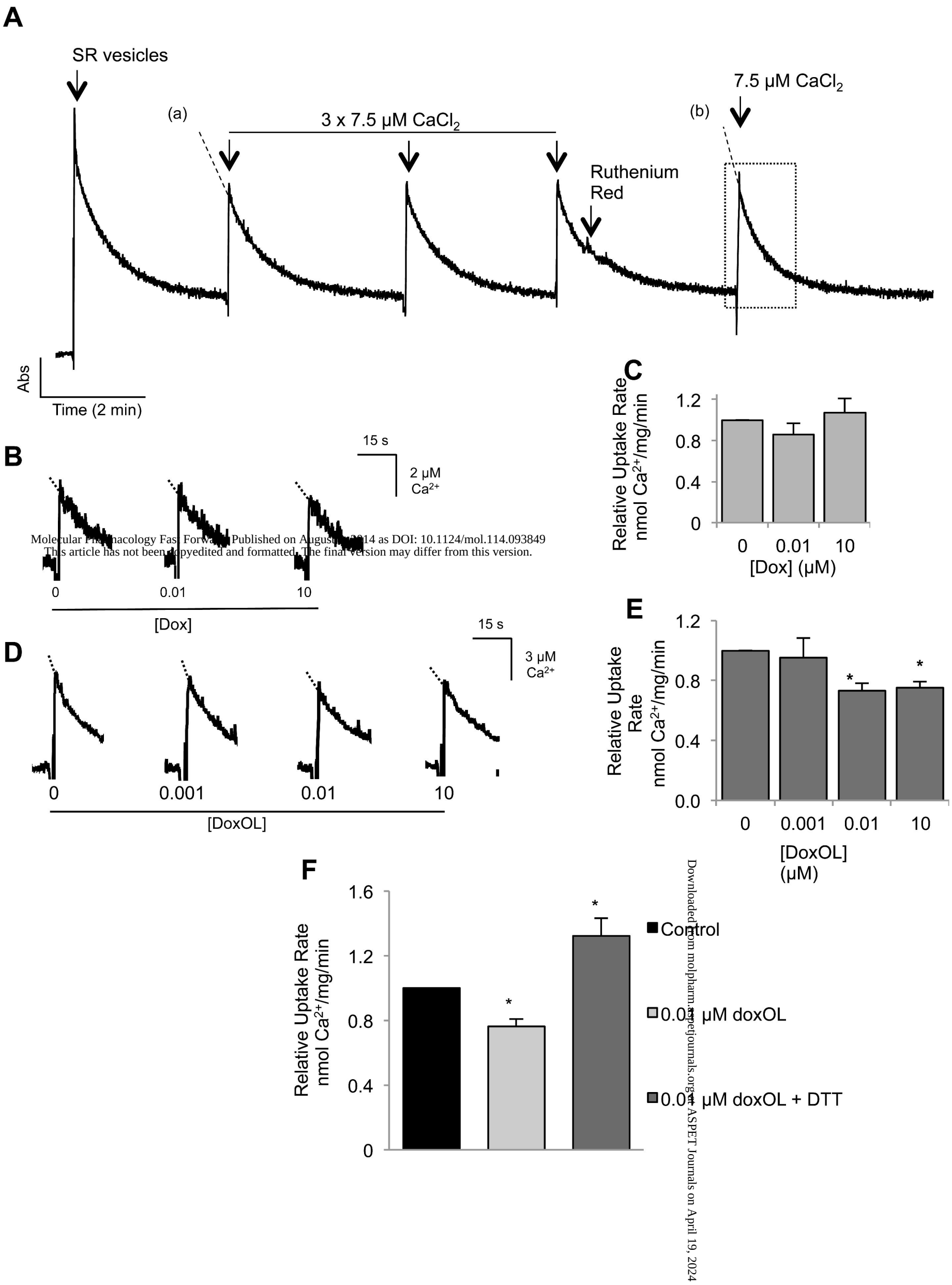

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Figure 7

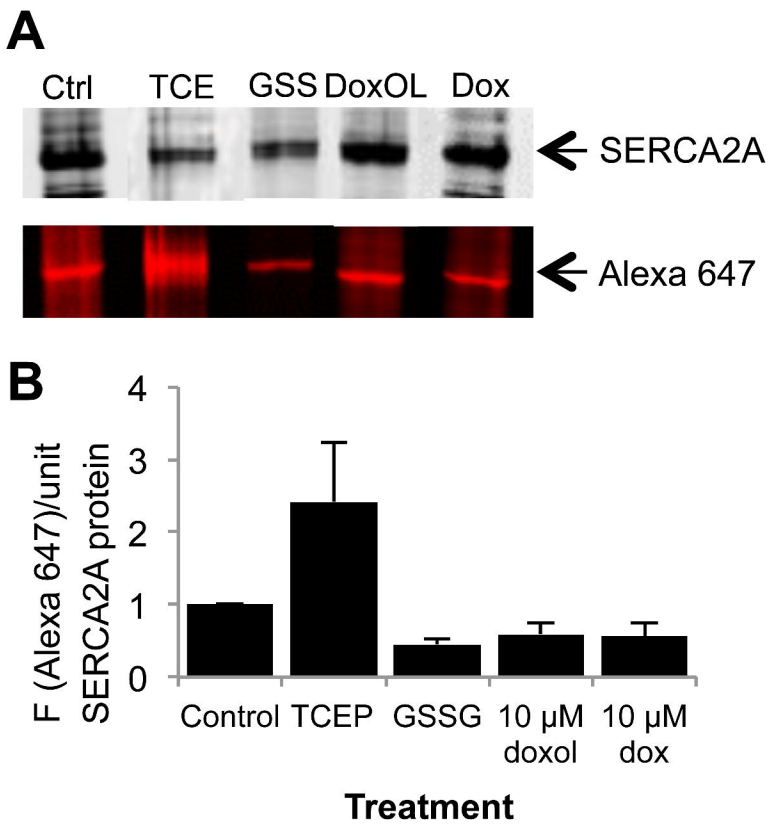

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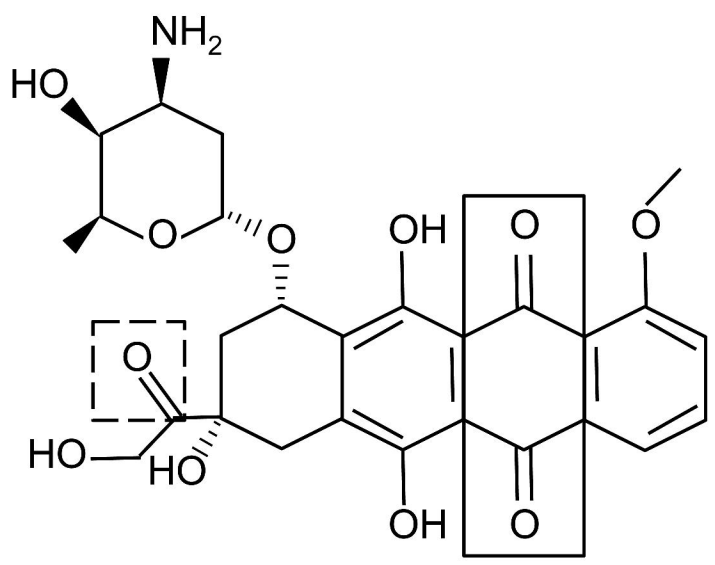
Figure 8

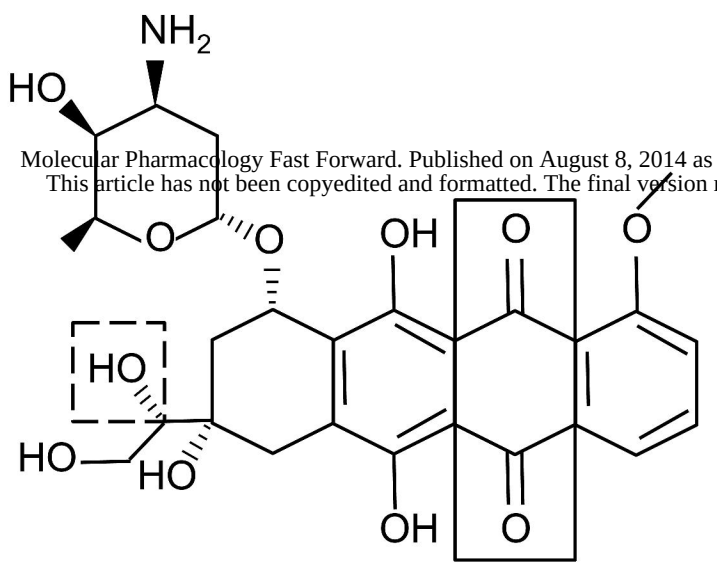
A

Doxorubicin



B

Doxorubicinol



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