Doxorubicin induces de novo expression of N-terminal truncated MMP-2 in cardiac myocytes

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Doxorubicin induces de novo expression of N-terminal truncated MMP-2 in cardiac myocytes

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Running Head: Doxorubicin activates intracellular MMP-2 in cardiac myocytes
Abstract

Anthracyclines, such as doxorubicin, are commonly prescribed antineoplastic agents which cause irreversible cardiac injury. Doxorubicin cardiotoxicity is initiated by increased oxidative stress in cardiomyocytes. Oxidative stress enhances intracellular matrix metalloproteinase-2 (MMP-2) by direct activation of its full length isoform and/or de novo expression of an N-terminal truncated (NTT-MMP-2) isoform. As MMP-2 is localized to the sarcomere we tested whether doxorubicin activates intracellular MMP-2 in neonatal rat ventricular myocytes (NRVM) and if it thereby proteolizes two of its identified sarcomeric targets, α-actinin and troponin I. Doxorubicin increased oxidative stress within 12 hr as indicated by reduced aconitase activity. This was associated with a twofold increase in MMP-2 protein levels and threefold higher gelatinolytic activity. MMP inhibitors ARP-100 or ONO-4817 (1 μM) prevented doxorubicin-induced MMP-2 activation. Doxorubicin also increased the levels and activity of MMP-2 secreted into the conditioned media. Doxorubicin upregulated the mRNA expression of both full length MMP-2 and NTT-MMP-2. α-Actinin levels remained unchanged, whereas doxorubicin downregulated troponin I in an MMP-independent manner. Doxorubicin induces oxidative stress and stimulates a robust increase in MMP-2 expression and activity in NRVM, including NTT-MMP-2. The sarcomeric proteins α-actinin and troponin I are, however, not targeted by MMP-2 under these conditions.

Key Words: doxorubicin, oxidative stress, matrix metalloproteinase, cardiac myocyte, sarcomere
1. Introduction

The therapeutic utility of many anticancer drugs, including anthracyclines such as doxorubicin (DXR), is hampered by their serious cardiotoxic side effects (Singal and Iliskovic 1998; Bloom et al. 2016). Current efforts to prevent heart failure by lowering the lifetime cumulative dose of DXR compromise cancer therapy and fail to prevent heart injury, as the drug can damage cardiac myocytes even after a single dose (Unverferth et al. 1983). Consequently, new strategies are needed to prevent heart injury in DXR chemotherapy. However, few, if any, have been successful because the mechanism by which DXR injures cardiac myocytes remains incompletely understood.

The detrimental effects of DXR on cardiac myocytes are believed to be primarily initiated by increased oxidative stress through the production of reactive oxygen and nitrogen species (Singal and Iliskovic 1998; Mukhopadhyay et al. 2009; Zhang et al. 2012). However, several studies using drugs to reduce oxidative stress have either failed to prevent DXR cardiotoxicity or increase the risk of secondary malignancies (Myers et al. 1983; Martin et al. 2009; Sawyer 2013), likely due to their inability to target specific reactive oxygen and nitrogen species or additional mechanisms involved in myocardial dysfunction. DXR cardiotoxicity is also associated with loss of sarcomeric proteins (Ito et al. 1990; Herman et al. 2001; Lim et al. 2004), impaired Ca\(^{2+}\) homeostasis (Jiang et al. 1994; Wang and Korth 1995), and mitochondrial dysfunction (Green and Leeuwenburgh 2002; Ichikawa et al. 2014). These adverse effects may be consistent with the consequences of activating matrix metalloproteinase-2 (MMP-2), an abundant extra- and intracellular protease in the heart (Hughes and Schulz 2014). In fact, a single dose of DXR was reported to increase matrix metalloproteinase-2 (MMP-2) protein levels in adult rat hearts (Bai et
al. 2004). However, the expression and activity of intracellular MMP-2 isoforms in DXR-exposed cardiac myocytes have not been elucidated and their proteolytic targets are unknown.

Full length MMP-2 (72 kDa) is a zinc-dependent protease involved in many physiological and pathological processes in the heart (Spinale 2007; Hughes and Schulz 2014). MMP-2 activity was initially found to directly contribute to heart failure and injury (Peterson et al. 2001; Spinale 2002; DeCoux et al. 2014) by remodeling extracellular matrix proteins. Cardiac specific overexpression of MMP-2 in mice caused severe systolic dysfunction, myofibrilysis, fibrosis, and cardiac myocyte dropout (Bergman et al. 2007). However, subsequent discovery of the subcellular localization of at least two intracellular MMP-2 isoforms in cardiac myocytes expanded its pathological roles, particularly in oxidative stress-induced heart injury (Wang et al. 2002). Full length MMP-2 can be activated by peroxynitrite via S-glutathiolation of a critical cysteine residue in its N-terminal propeptide domain (Viappiani et al. 2009). Oxidative stress also triggers the transcription of an alternative promoter in the first intron of the *Mmp2* gene to generate NTT-MMP-2 which is devoid of a signal sequence and part of its inhibitory propeptide domain, rendering it proteolytically active (Lovett et al. 2012). NTT-MMP-2 was first discovered in cardiac myocytes subjected to hypoxia and is primarily mitochondrial (Lovett et al. 2012; Lovett et al. 2013). Mice with cardiac-specific expression of NTT-MMP-2 developed progressive heart failure not involving myofibrilysis but myocardial inflammation and cell death (Lovett et al. 2013). Whether DXR triggers expression of NTT-MMP-2 in cardiac myocytes is unknown.

Activation of intracellular MMP-2 plays an important role in pathophysiology of oxidative stress-induced heart injury as it is able to proteolyze many specific sarcomeric proteins, including
troponin I (Wang et al. 2002), myosin light chain-1 (Sawicki et al. 2005), α-actinin (Sung et al. 2007), and titin (Ali et al. 2010). Cleavage of sarcomeric proteins by MMP-2 contributes to acute cardiac contractile dysfunction in myocardial ischemia-reperfusion injury (Wang et al. 2002). MMP inhibitors improved the recovery of cardiac contractile function by preventing the degradation of sarcomeric proteins (Cheung et al. 2000; Wang et al. 2002; Ali et al. 2010). In addition to the sarcomere, MMP-2 is also localized to the cytoskeleton (Sung et al. 2007), nuclei (Baghirova et al. 2016), caveolae (Chow et al. 2007), mitochondria (Lovett et al. 2012; Hughes et al. 2014), and the mitochondria-associated membrane (MAM) (Hughes et al. 2014).

In the present study we describe DXR-induced activation of full length MMP-2 and de novo expression of NTT-MMP-2 in cardiac myocytes. Despite the upregulation of two distinct intracellular MMP-2 isoforms, this did not result in the proteolysis of α-actinin or troponin I.

2. Methods

All experiments involving animals in this study were approved by the University of Alberta Institutional Animal Care and Use Committee, in accordance to the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care (CCAC).

2.1 Cell culture

NRVM were isolated from 1-2 day-old Sprague-Dawley rats as previously described (Fan et al. 2016). NRVM from 10 pups were pooled and plated (1.2×10^5 cells/cm^2) in DMEM/F-12 (Thermo Fisher Scientific) containing 10% fetal bovine serum. Cells were maintained at 37°C for 2 days prior to experiments. HT1080 cells (a human fibrosarcoma cell line) were cultured in
DMEM (Thermo Fisher Scientific) containing 10% fetal bovine serum at 37°C.

2.2 Experimental Protocol

All cell experiments were conducted under serum-free conditions. Cells were washed with phosphate buffered saline and the medium was replaced with serum-free DMEM/F12. Stock solutions of the selective MMP-2 inhibitor ARP-100 (Cayman Chemical, in DMSO), the gelatinases (MMP-2 and MMP-9) selective inhibitor ONO-4817 (a gift from Ono Pharmaceutical, in DMSO), and doxorubicin hydrochloride (DXR, Sigma-Aldrich) were prepared (10, 10, and 3.45 mM, respectively). NRVM and HT1080 cells were treated with DMSO (vehicle, maximum 0.01% v:v) or DXR (0.5 µM) with or without MMP inhibitors ARP-100 or ONO-4817 (1 µM, each). Cells were incubated at 37°C for 2, 6, 12, or 24 hr. Cell conditioned media were collected to measure cell viability and levels of secreted MMP-2. Cells were lysed with RIPA buffer (pH 8.0) containing 1% proteinase inhibitor cocktail (Sigma-Aldrich) for 5 min at 4°C. Cell lysates were centrifuged (10,000 g, 10 min, 4°C) to remove cellular debris. Total lysate protein concentration was measured (bicinchoninic acid assay, Sigma-Aldrich) using bovine serum albumin as a standard. MMP-2 activity in cell lysates and conditioned media were measured by gelatin zymography as previously described (Sung et al. 2007).

2.3 Cell viability

Lactate dehydrogenase (LDH) activity was measured in freshly collected conditioned media following centrifugation (10,000 g, 10 min, 4°C) as a marker of cell necrosis using a CytoTox-ONE Homogeneous Membrane Integrity assay kit (Promega) and a Synergy H4 multi-well fluorescence plate reader (BioTek, Winooski, VT, USA).
2.4 Cellular oxidative stress by aconitase activity

Aconitase, a mitochondrial enzyme that converts citrate to isocitrate, is inactivated directly by reactive oxygen-nitrogen species including peroxynitrite (Hausladen and Fridovich 1994) and thereby was used to estimate oxidative stress (Hausladen and Fridovich 1994; Csont et al. 2005). Aconitase activity from NRVM lysates was measured spectrophotometrically using a microplate assay kit (Abcam, Cambridge, UK) which measures absorbance upon formation of the intermediate cis-aconitate from citrate. In brief, 10 µg of cell lysate protein diluted in aconitase preservation solution was incubated with 200 µL of assay buffer at room temperature for 30 min. The increase in absorbance at 240 nm was measured over 30 min. Aconitase activity was determined by the difference in optical density before and after the incubation divide by the incubation time.

2.5 Western blot analysis

Cell lysate protein aliquots (10 µg each) were separated on 10% polyacrylamide gels by electrophoresis. Proteins were transferred onto polyvinylidene difluoride membranes (Bio-Rad). Membranes were immunoblotted with primary monoclonal antibodies against α-actinin (MAB1682, EMD Millipore), MMP-2 (ab92536, Abcam), TIMP-3 (sc-30075, Santa Cruz, Dallas, TX, USA), TIMP-4 (ab58425, Abcam), troponin I (MA1040, iPOC, Toronto, ON, Canada), and GAPDH (2118S, Cell Signaling Technology, Danvers, MA, USA). Primary antibodies were then probed with the appropriate secondary antibody, either goat anti-rabbit (CLCC42007, Cedarlane) or goat anti-mouse (CLCC30007, Cedarlane). Protein bands were visualized using chemiluminescent detection reagent (Amersham ECL Prime, GE Healthcare) and exposed to autoradiography film. These were scanned with a Bio-Rad GS-800 densitometer and quantified on
2.6 RNA quantification by quantitative PCR

Total RNA was extracted from NRVM using TRIzol (Invitrogen) according to the manufacturer’s instructions. RNA quality and concentration was assessed using a NanoDrop ND8000 spectrophotometer (Thermo Fisher Scientific). Reverse transcription was performed after DNase I treatment, using SuperScript II Reverse Transcriptase and Oligo(dT) (Invitrogen); controls where reverse transcriptase was omitted were included to confirm absence of genomic DNA contamination. Samples were processed in triplicate by qPCR using SYBR Green I Master Mix (Roche Diagnostics) in a LightCycler 480 System (Roche Diagnostics) with 40 cycles (30 sec each of denaturation, annealing, and extension). $C_q$ values and melt curves were analyzed with LightCycler 480 software v1.5.1.

Primers were designed and optimized for *Rattus norvegicus* using the PrimerQuest Tool (Integrated DNA Technologies). Sequences for the designed primers and amplicon sizes are presented in Table 1. *Gapdh* was used as a reference gene. To generate standards for quantification we obtained PCR products by endpoint PCR and purified them from agarose gels using the NucleoSpin Gel & PCR Clean-up Kit (Macherey-Nagel, Düren, Germany). Purified products were quantified and sequenced. As standards for qPCR, we used serial log$_{10}$ dilutions ranging from $1 \times 10^2$ to $1 \times 10^6$ copies of each PCR product. qPCR target $C_q$ values were used to calculate copy numbers using the standard curve, normalized against *Gapdh*, and are reported as fold-copy numbers compared to *Gapdh*. Total *Mmp2* mRNA expression, which includes full length MMP-2 and NTT-MMP-2, was measured using primers which target common exons 2-3 in *Mmp2*. *Mmp2*
mRNAs encoding full length MMP-2 (including exon 1, absent in NTT-MMP-2), were quantified using intron-spanning primers which target exons 1-2. NTT-\textit{Mmp2} expression was calculated as the difference in copy numbers between \textit{Mmp2} exons 2-3 and \textit{Mmp2} exons 1-2 (i.e., total MMP-2 minus full length MMP-2 expression).

2.7 Statistical analysis

An individual result (a single biological replicate) corresponds to the NRVM cultured from a single isolation of cells pooled from the hearts of 10 pups. Results are expressed as mean ± S.E.M. and were analyzed for statistical comparison with Prism 7.01 (GraphPad Software), using one-way ANOVA followed by Tukey’s post-hoc test or two-way ANOVA followed by Bonferroni post-hoc test, as appropriate. \(p<0.05\) was used as the criterion for statistical significance.

3. Results

3.1 DXR preferentially induces necrosis in tumor cells but not NRVM and enhances oxidative stress

We used a concentration of DXR (0.5 \(\mu\)M) which is within the concentration range seen in the blood 6 hr after administrating DXR in chemotherapy patients (Greene et al. 1983). To confirm that this concentration of DXR induced tumor cell death, we treated HT1080 cells with 0.5 \(\mu\)M DXR for 24 hr and measured cell necrosis by LDH release. DXR caused cell death in HT1080 cells after 24 hr compared to control, confirming that the concentration used kills tumor cells (Fig. 1A). In marked contrast, DXR did not cause NRVM cell death for up to 24 hr. However, 36 and 48 hr DXR exposure increased NRVM cell death to 15 and 50%, respectively (Fig. 1B). DXR
increased oxidative stress in NRVM, seen by a 57% and 62% reduction in aconitase activity after 12 and 24 hr, respectively (Fig. 1C).

3.2 DXR increases intracellular MMP-2 in NRVM lysates

We next examined the effect of DXR on intracellular MMP-2 protein level and activity detectable in NRVM lysates (in the absence of cell necrosis) using western blot and gelatin zymography, respectively. DXR increased MMP-2 protein levels in a time-dependent manner, up to 239 ± 20% of control after 12 hr, which remained elevated at 24 hr (Fig. 2A). MMP-2 activity from control cells appeared as a single 72 kDa band, with no evidence of other gelatinolytic activities, including 64 kDa MMP-2 (formed following proteolysis of 72 kDa MMP-2 by an MMP-14/TIMP-2 dependent process at the cell membrane) (Fig. 2B), or MMP-9. DXR treatment resulted in a significant, time-dependent increase in full length MMP-2 activity which also peaked at 12 hr (311 ± 40% of control, and remained elevated at 24 hr (287 ± 30% of control) (Fig. 2B). The MMP inhibitors ARP-100 or ONO-4817 had no effect on MMP-2 protein levels in control and DXR-exposed cells (Fig. 2C). Neither ARP-100 nor ONO-4817 affected activity in control cells but significantly attenuated the DXR-induced increase in MMP-2 activity (Fig. 2D).

3.3 DXR increases levels and activity of secreted MMP-2

To determine whether DXR alters the abundance of secreted MMP-2, MMP-2 levels and activity in the conditioned media were measured by western blot and gelatin zymography, respectively. DXR increased secreted MMP-2 protein levels by nearly fourfold (Fig. 3A). This increase in protein levels corresponded to an increase in MMP-2 activity by over fourfold (Fig. 3B). As expected, MMP inhibitors ARP-100 or ONO-4817 did not affect the abundance in secreted...
MMP-2 protein levels but attenuated secreted MMP-2 activity to control (Fig. 3A,B). Activity by a lower molecular weight gelatinase was also detected in the conditioned media. However, this gelatinolytic activity was unchanged between groups. This secreted ~35 kDa gelatinase is likely the catalytic domain of MMP-2, given that there is no detectable MMP-9 activity in the conditioned media.

3.4 DXR increased NTT-MMP-2 and full length MMP-2 mRNA levels

To better understand which intracellular MMP-2 isoforms are activated by DXR, we measured the effect of DXR on total \textit{Mmp2}, full length \textit{Mmp2} and NTT-\textit{Mmp2} mRNA copy numbers. DXR increased total \textit{Mmp2} mRNA levels in a time-dependent manner, up to 580% of control after 24 hr (Fig. 4A). 24 hr DXR exposure increased full length \textit{Mmp2} and NTT-\textit{Mmp2} mRNA levels by 680% and 560%, respectively (Fig. 4B,C). By comparing the copy numbers of full length \textit{Mmp2} and NTT-\textit{Mmp2} to total \textit{Mmp2}, we determined the proportion of each MMP-2 isoform that constitute total MMP-2. In both control and DXR-treated NRVM, 88% of total \textit{Mmp2} is expressed as the NTT-MMP-2 isoform and the remaining 12% is expressed as full length MMP-2 (Fig. 4D).

3.5 Potential sarcomeric targets of MMP-2 in NRVM

In adult rat hearts undergoing oxidative stress injury, intracellular MMP-2 proteolizes the sarcomeric proteins troponin I (Wang et al. 2002) and \(\alpha\)-actinin (Sung et al. 2007), thereby impairing cardiac muscle contractility (Wang et al. 2002). We assessed the levels of these proteins in NRVM exposed to DXR for 24 hr. DXR treatment had no effect on \(\alpha\)-actinin levels (Fig. 4A), whereas it caused a 40% decrease in troponin I which was not prevented by ARP-100 or ONO-
4817 (Fig. 5B). Lower molecular weight bands, suggestive of proteolytic cleavage, were not detected for either protein. ARP-100 or ONO-4817 alone had no effect on troponin I (Fig. 5B). To further understand the discrepancy between the effects of MMP inhibitors and troponin I levels, we measured the levels of TIMP-4 in cell lysates, an intracellular TIMP isoform found in cardiomyocytes (Schulze et al. 2003). However TIMP-4 levels were not affected by DXR with or without MMP inhibitors (Fig. 5C). TIMP-3, the primary extracellular TIMP isoform, was not detectable in the NRVM lysates (data not shown). Troponin I mRNA (Tnni3) expression was then evaluated by qPCR. 24 hr DXR treatment nearly abolished Tnni3 expression by 95% relative to control cells (Fig. 5D). These results indicate that the DXR-induced decrease in troponin I in NRVM occurs at a transcriptional level, and that, under these conditions, the DXR-induced up-regulation of MMP-2 does not directly result in increased troponin I proteolysis.

4. Discussion

In this study, we demonstrated that DXR used at a clinically relevant concentration increases intracellular MMP-2 protein levels and activity detectable in neonatal cardiac myocytes. For the first time we identified the intracellular MMP-2 isoforms stimulated by DXR in cardiac myocytes as full length MMP-2 was upregulated and accompanied by the de novo expression of NTT-MMP-2. In contrast to our hypothesis that MMP-2 would proteolyze sarcomeric proteins α-actinin and/or troponin I, we found no changes in the former while the reduction in troponin I was independent of MMP-2 activity.

DXR enhanced oxidative stress in NRVM, measured as a reduction in aconitase activity, was concurrent with increased MMP-2 activity in cell lysates. DXR enhances the generation of...
reactive oxygen-nitrogen species including peroxynitrite in cardiac myocytes (Mukhopadhyay et al. 2009) and myocardium (Weinstein et al. 2000). Oxidative stress activates intracellular MMP-2 by three primary mechanisms. Firstly, peroxynitrite reacts with full length MMP-2 which causes the S-glutathiolation of a critical cysteine residue in its autoinhibitory domain (Viappiani et al. 2009). This post-translational modification exposes the catalytic zinc site and renders the enzyme proteolytically active (Viappiani et al. 2009). Secondly, oxidative stress also increases the biosynthesis of full length MMP-2 by enhancing the binding of AP-1 to the \textit{Mmp2} gene promoter (Alfonso-Jaume et al. 2006). Thirdly, it also can trigger the \textit{de novo} expression of NTT-MMP-2 via an alternate transcription start site located within the first intron (Lovett et al. 2012). We found that DXR increased the mRNA expression for both full length \textit{Mmp2} and NTT-\textit{Mmp2}. Our results reveal that DXR increases MMP-2 activity in cardiac myocytes by upregulating these two intracellular isoforms. Interestingly, NTT-\textit{Mmp2} constituted almost 90\% of total \textit{Mmp2} expression in both control and doxorubicin treated cells. It remains unclear why control NRVM express NTT-MMP-2. It is likely that the harsh process of isolating NRVMs via enzymatic digestion followed by serum starvation during cell treatment enhances cellular oxidative stress even in control cells.

In addition to increased MMP-2 activity detectable in NRVM lysates, DXR also increased secreted 72 kDa MMP-2 levels and activity in the conditioned media. We did not detect 64 kDa MMP-2 in the conditioned media, suggesting that MMP-2 was not proteolytically activated by MMP-14/TIMP-2 in the extracellular matrix. This is consistent with our previous work which showed that increased oxidative stress (by ischemia-reperfusion injury) stimulates 72 kDa MMP-2 secretion in isolated and perfused rat hearts (Cheung et al. 2000). Increased secretion of MMP-
2 likely contributes to adverse myocardial extracellular matrix remodeling in chronic DXR cardiotoxicity (Octavia et al. 2012).

Increased intracellular MMP-2 activity and myofibrillar disorganization occur in several heart pathologies associated with increased oxidative stress (Wang et al. 2002; Sung et al. 2007; Ali et al. 2010). In myocardial ischemia-reperfusion injury, MMP-2 localized to the Z-disc region of the sarcomere (Ali et al. 2010) proteolyzes sarcomeric proteins including troponin I (Wang et al. 2002), α-actinin (Sung et al. 2007), and titin (Ali et al. 2010). DXR cardiotoxicity is associated with a loss of sarcomeric proteins including α-actin, titin, troponin I, myosin light chain 2, and myosin binding protein C (Ito et al. 1990; Lim et al. 2004; Aryal et al. 2014). However, it is unknown whether this loss occurs at a transcriptional level or post-translationally via proteolysis. DXR caused a reduction in cellular troponin I without detectable degradation products which was not attenuated with two different selective MMP inhibitors. Although troponin I has been identified as a MMP-2 substrate in myocardial ischemia-reperfusion injury in adult rat hearts, it is possible that MMP-2 is not localized to troponin I in NRVM exposed to DXR. Recent work has determined that NTT-MMP-2 is, in part, localized to subsarcolemmal mitochondria. Given that 90% of the MMP-2 mRNA expressed in NRVM is NTT-MMP-2, our findings that MMP inhibitors did not prevent DXR-induced reduced troponin I levels are consistent with previous reports that upregulation of NTT-MMP-2 does not result in myofibrilysis (Lovett et al. 2014). To test whether DXR triggers compensatory TIMP expression, we measured the levels of TIMP-4, the most abundant TIMP in the heart (Leco et al. 1997) and which is localized within cardiomyocytes (Schulze et al. 2003), in NRVM lysates. However TIMP-4 levels were unaffected by DXR in the presence or absence of MMP inhibitors, ruling out the possibility that enhanced intracellular TIMP
may have blocked troponin I and α-actinin proteolysis by MMP-2. Instead, we found that a
significant reduction in troponin I mRNA may account for its reduced protein level. Overall, our
results suggest the loss of troponin I caused by DXR in NRVM is due to transcriptional down-
regulation, and not proteolysis.

There are limitations to our study. To understand mechanisms related to anthracycline
cardiotoxicity in adults, cardiac myocytes isolated from adult animals may be preferable for study.
However, they are more unstable than NRVM in culture and their sarcomeric integrity is
compromised over time. Despite NRVM being more robust in culture and showing spontaneous
contractile activity, they are immature cells which exhibit disorganized myofibrils (Peter et al.
2016) and express both slow skeletal and cardiac troponin I isoforms (Hunkeler et al. 1991;
Murphy et al. 1991). Whether MMP-2 localizes to the thin myofilaments of NRVM and is able to
proteolyze slow skeletal troponin I or other sarcomeric proteins needs further study.

In conclusion, we have shown that the anthracycline DXR activates full length MMP-2 and
triggers increased expression of NTT-MMP-2 in neonatal rat cardiac myocytes. DXR-induced
activation of MMP-2 was successfully attenuated by selective MMP inhibitors. We found that
troponin I protein levels were reduced in an MMP-independent manner as a result of
transcriptional down-regulation. Given the known ability of MMP-2 to degrade intracellular
proteins with significant pathophysiological consequences, it will be important to identify the roles
and proteolytic targets of MMP-2 at its subcellular locales in anthracycline cardiotoxicity. For
example, in addition to the sarcomere, MMP-2 and NTT-MMP-2 are also compartmentalized in
the mitochondria, mitochondria-associated membrane, and nucleus (Lovett et al. 2012; Hughes et

**Disclosures**

None

**Acknowledgements**

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References:


Figure legends

Figure 1 – Effects of doxorubicin on cell viability and oxidative stress. (A) DXR (0.5 µM, 24 hr) induced cell death in HT1080 human fibrosarcoma cells but not in NRVM (n=8). (B) DXR (0.5 µM) caused significant cell death in NRVM only after 36 hr or longer exposure (n=5). (C) DXR significantly enhanced cellular oxidative stress in NRVM at 12 and 24 hr, as measured by a reduction in aconitase activity (n=4). Bar graphs represent mean ± S.E.M. * p<0.05 versus vehicle control.

Figure 2 – Doxorubicin increases MMP-2 activity and protein level in NRVM lysates. (A) DXR (0.5 µM) increased MMP-2 protein level in NRVM lysates in a time-dependent manner, peaking at 12 hr (n=5). (B) DXR increased intracellular MMP-2 activity in a time-dependent manner (n=5). (C) MMP inhibitors ARP-100 and ONO-4817 (1 µM each) did not affect MMP-2 protein levels (n=5). (D) ARP-100 and ONO-4817 attenuated DXR-induced increase in MMP-2 activity (n=7). Std: HT-1080 cell conditioned medium. Bar graphs represent mean ± S.E.M. * p<0.05 versus vehicle control.

Figure 3 – Doxorubicin increases secreted MMP-2 levels and activity. (A) DXR (0.5 µM, 24 hr) increased levels of MMP-2 secreted into the conditioned media (n=4). (B) DXR increased secreted MMP-2 activity, an effect that was reduced by ARP-100 or ONO-4817 (n=5). ABU: arbitrary units. Bar graphs represent mean ± S.E.M. * p<0.05, versus control.

Figure 4 – Doxorubicin enhances the expression of full length and N-terminal truncated Mmp2 mRNA. (A) DXR (0.5 µM) increased expression of total Mmp2 (A), as well as full length (72 kDa) Mmp2 (B), and NTT-Mmp2 (C) isoforms in a time-dependent manner as measured by

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qPCR. (D) The majority of DXR-induced $Mmp2$ expression corresponded to the NTT-MMP-2 isoform rather than the full length MMP-2 isoform (n=6-8). Bar graphs represent mean ± S.E.M. * $p<0.05$ versus vehicle control.

**Figure 5** – Effect of doxorubicin on levels of sarcomeric proteins in NRVM. (A) DXR (0.5 μM) did not affect $\alpha$-actinin levels after 24 hr exposure (n=5). (B) In contrast, DXR reduced the levels of troponin I which was not prevented with MMP inhibitors ARP-100 or ONO-4817 (n=5). (C) DXR did not alter TIMP-4 protein levels (n=6). (D) DXR reduced troponin I ($Tnni3$) mRNA expression after 24 hr (n=8). Bar graphs represent mean ± S.E.M. * $p<0.05$ versus vehicle control.
Table 1

Table 1: Primer sequences for qPCR.

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Figure 1

A

Cell death (%) vs. NRVM and HT1080.

B

Cell death (%) vs. NRVM with DXR treatment over time.

C

Aconitase activity (mOD/min) vs. C and DXR treatment at 12 and 24 hr.
Figure 2

A

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MMP-2

| GAPDH |

37 kDa

MMP-2

B

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MMP-2

| MMP-2 activity (fold) |

MMP-2

C

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MMP-2

| GAPDH |

37 kDa

MMP-2

D

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MMP-2

| MMP-2 activity (fold) |

MMP-2

https://mc06.manuscriptcentral.com/cjpp-pubs
Figure 3

A

![Image of Western blot showing MMP-2 levels at 72 kDa with DXR (24 hr) treatment.](image)

B

![Image of Western blot showing MMP-9 and MMP-2 levels at 92 kDa, 72 kDa, and 35 kDa with DXR (24 hr) treatment.](image)
Figure 4

A

Total *Mmp2*

B

Full length-*Mmp2*

C

NTT-*Mmp2*

D

Proportion of MMP-2 isoforms
**Figure 5**

**A**

- **DXR**
  - C  |  ARP  |  ONO
  - 100 kDa
  - 37 kDa
- **α-actinin**
  - GAPDH

**B**

- **DXR**
  - C  |  ARP  |  ONO
  - 25 kDa
  - 37 kDa
- **troponin I**
  - GAPDH

**C**

- **DXR**
  - C  |  C  |  ARP  |  ONO
  - 25 kDa
  - 37 kDa
- **TIMP-4**
  - GAPDH

**D**

- **Tnni3**
  - Fold change
  - 12 hr  |  24 hr
  - Control  |  DXR