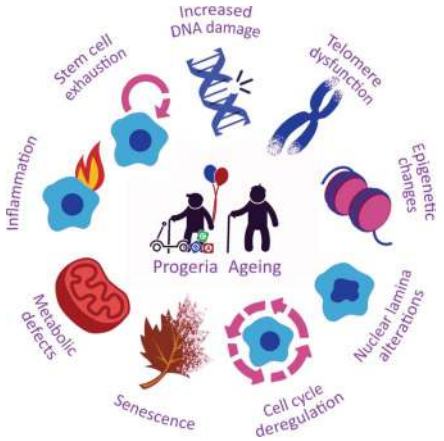
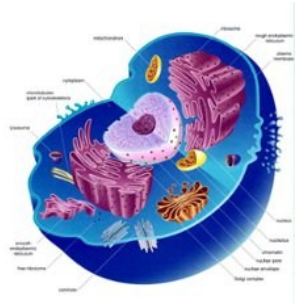
Showcase of Medical Discoveries:

Basic Subcellular Mechanisms





Wednesday, November 9, 2016

4:30—6:00 p.m.

A Wine & Cheese Reception Featuring UAMS Investigators Discussing their Research and Discoveries.

Winthrop P. Rockefeller Cancer Institute
10th Floor Rotunda

UAMS
COLLEGE OF MEDICINE
UNIVERSITY OF ARKANSAS FOR MEDICAL SCIENCES

UAMS Office of Research

Poster #1

New Role of Sirt2 in Alleviating Chemotherapy-induced Peripheral Neuropathy Pain

Manchao Zhang, Wuying Du, Hao Yu, Shengkai Kin, Ju Huwan, Cho,
Parmeet K. Manchada, Rahman Mohammad, and Fen Xia

Chemotherapy-induced peripheral neuropathy (CIPN) is one of the major health concerns for cancer patients. The natural compound, resveratrol is used to alleviate neuropathy pain in diabetics through activating the NAD -dependent deacetylase, Sirt2. We are interested to establish the role of Sirt2 in CIPN. We have established mouse models of cisplatin-induced neuropathy in Sirt2-overexpressing (Sirt2 knock-in), Sirt2-deficient (Sirt2 KO), and their wild type control, C57BL/6 mice using the dose similar to that used in patients. Adult mice were treated with cisplatin 2.3 mg/kg daily intraperitoneal injection for 5 days (1 cycle), followed by 5 days of rest and another cycle of cisplatin treatment. Behavioral evaluations included von Frey and radiant heat tests at baseline and at weekly intervals. Following two treatment cycles, mice in the cisplatin treatment group demonstrated significant higher level of mechanical allodynia compared to vehicle control group for C57BL/6 mice, this difference was even more significant for Sirt2 KO mice. However, there is no such difference for Sirt2 KI mice. We further recapitulated this observation in Lewis lung tumor bearing Sirt2 KI and C57BL/6 mice. These findings support the role of Sirt2 in protecting peripheral nerves from CIPN. Our ongoing effort is to identify Sirt2 deacetylation targets that modulate this function.

Poster #14

Protein Aggregates, Proteasomes, and Autophagosomes: Keys to Understanding Neurodegeneration

Sundaram Balasubramaniam, Srinivas Ayyadevara,
and Robert Shmookler Reis

Age dependent neurodegenerative diseases (Alzheimer's, Parkinson's and Huntington's) feature neurotoxic aggregates diagnostic of each disease—inclusions containing misfolded, oxidized, and hyperphosphorylated proteins. Many aggregate proteins are shared among diverse neurodegenerative diseases, suggesting that aggregation is non-random. Beyond previously characterized "seed" proteins, additional shared proteins may play unknown roles in aggregate progression and neurotoxicity. A key hallmark of neurodegenerative diseases is disruption of protein-homeostasis mechanisms, particularly the ubiquitin/proteasome system (UPS) and autophagy. Aberrant and misfolded proteins that escape degradation due to UPS failure may coalesce into "indigestible" aggregates resistant to autophagy. Ubiquitinated proteins are enriched in many neurodegenerative disease inclusions, consistent with failure of protein degradation systems, but the underlying mechanisms remain elusive. In recent publications, we identified key proteins that block protein degradation by forming tight complexes with ubiquitinated substrates. In both *C. elegans* and human neuroblastoma (cell-culture) models of neurodegeneration, knockdown of these proteins reduces the accumulation of aggregates and ubiquitinated constituents. Computational modeling of functionally implicated proteins predicts tight interaction with polyubiquitins. Our strategy, combining animal models, analysis of patient tissue samples, and *in silico* simulations, has laid the foundation for defining the aggregate interaction network and the mechanisms involved in age-dependent failure of protein homeostasis leading to aggregation.

Poster #13

Increased stability of mitochondrial CYP2E1 against degradation compared to microsomal CYP2E1

Jessica H. Hartman, Andres A. Caro, Martin J. Ronis, and Grover P. Miller

Steady-state CYP2E1 levels impact its bioactivation of drugs and pol-lutants to reactive metabolites that damage DNA and proteins. The balance between CYP2E1 synthesis and degradation is well known for microsomal CYP2E1 localized to the endoplasmic reticulum, but not mitochondrial CYP2E1. Consequently, we assessed CYP2E1 stability through a cycloheximide chase assay using cell lines preferentially expressing either microsomal (E47) or mitochondrial (mE10) CYP2E1. Corresponding subcellular fraction contained catalytically active CYP2E1 and lacked any cross contamination. After blocking protein synthesis, we measured the decay of CYP2E1 protein levels by Western blot over 24 hr. The microsomal CYP2E1 half-life was 12 hr, as reported by others. By contrast, mitochondrial CYP2E1 levels did not decrease after 24 hr. For a control, we demonstrated mitochondrial SDHA similarly lacked a decrease in levels (half-life 4 d). These findings indicate that mitochondrial CYP2E1 is significantly more stable than microsomal CYP2E1, which could impact its regulation of expression and contributions to metabolism. First, mitochondrial CYP2E1 induction is not likely due to increased stabilization like microsomal CYP2E1. Second, once induced, elevated levels of mitochondrial CYP2E1 will persist and thus contribute to metabolism. Taken together, these outcomes would alter relative contributions of mitochondrial and microsomal CYP2E1 to metabolism as a function of time.

Poster #2

Investigation of Structure-activity Relationships for Small-Molecule Inhibitors of Human DNA Polymerase eta

.

Maroof K. Zafar, Amit Ketkar, Sarah Eddy, Leena Maddukuri, Narsimha R. Penthala, Peter A. Crooks, and Robert L. Eoff

Efficient DNA replication by high-fidelity polymerases (pols) can be perturbed by both endogenous and exogenous sources of DNA damage. Radiotherapy and many chemotherapeutic drugs rely upon DNA damage to limit tumor growth. These genotoxic agents can be rendered ineffective through direct bypass of the damage by translesion DNA pols, such as the Y-family member DNA pol eta (hpol η). By screening a library of novel compounds synthesized at UAMS, we have identified several inhibitors of hpol η. We went on to investigate the structure-activity relationships important for successful inhibition of hpol η and have elucidated the kinetic mechanism of action for the most potent compound identified to date, PNR-7-02. Futhermore, we have examined the specificity of PNR-702 against hpol η by testing the activity of compound against pols from the A-, B-, X- and Y-family. Finally, we identified another hpol η inhibitor, PNR-9-59, that exhibited similar potency and increased solubility compared to PNR-7-02. In summary, our work has improved the potency and drug-likeness of small-molecule inhibitors of hpol η, and it stands as the first detailed analysis of structure-activity relationships for inhibitors of a TLS pol involved in resistance to chemotherapeutics.

Poster #3

Exposure to Trichloroethylene Alters Epigenetic Profile in CD4+ T Cells

Kathleen M. Gilbert, Sarah J. Blossom, Brad Reisfeld, Kanan Vyas,
Craig A. Cooney, and Sudeepa Bhattacharyya

Exposure to solvent and water pollutant trichloroethylene (TCE) induces autoimmune hepatitis (AIH), and alters $CD4^+$ T cell function in mice. To define etiology, this study tested whether long-term exposure to TCE changed the methylome in $CD4^+$ T cells.

Female MRL+/+ mice were exposed to TCE in drinking water for 40 weeks. DNA isolated from effector/memory $CD4^+$ T cells was subjected to reduced representation bisulfite sequencing (RRBS). When a 25% difference between control and TCE samples was used as the cutoff, changes in 1,107 individual CpG sites, and 184 differentially-methylated regions (DMR) were identified.

Cumulatively, exposure to TCE dramatically changed the normal pattern from one in which most CpG sites were either hypo- or hyper-methylated to one in which CpG methylation averaged 50%. In terms of gene-specific effects, TCE decreased by 36.5% the methylation of a DMR near the TSS of *Cxxc1*. Transcriptomics showed an accompanying increase in *Cxxc1*. *Cxxc1* encodes for a protein that regulates both cytosine and histone methylation.

Taken together, the results suggested that TCE loosened the normally rigid constraints on CpG methylation in $CD4^+$ T cells, and moved methylation to a mid-range that is more likely to enable a change in gene expression. A TCE-induced decrease in the methylation of *Cxxc1* could help explain the cumulative effects of TCE, and help account for its immunotoxicity.

Poster #12

Novel Mechanism of Salt-sensitive Hypertension: CD8+ T Cells Stimulate Sodium Chloride Co-transporter in Kidney

Yunmeng Liu, Tonya M Rafferty, Sung W Rhee, Jessica S Webber,
Beixiang He, Shengyu Mu*

Recent studies suggest a role for T lymphocytes in hypertension. However, whether T cells contribute to renal sodium retention and salt-sensitive hypertension is unknown. Here we demonstrate that T cells infiltrate into the kidney of salt-sensitive hypertensive animals. In particular, CD8+ T cells directly contact the distal convoluted tubule (DCT) in the kidneys of DOCA-salt mice and CD8+ T cell-injected mice, leading to up-regulation and activation of sodium chloride co-transporter (NCC, p-NCC) and the development of salt-sensitive hypertension. Co-culture with CD8+ T cells upregulates NCC in mouse DCT cells via ROS-induced activation of Src kinase, up-regulation of the K+ channel Kir4.1, and stimulation of the Cl- channel ClC-K. The later event increases chloride efflux, leading to compensatory chloride influx via NCC activation at the cost of increasing sodium retention. Collectively, these findings provide a novel mechanism for adaptive immunity involvement in the kidney defect in sodium handling, which contributes to the pathogenesis of salt-sensitive hypertension.

Poster #11

Sc65-null Mice Provide Evidence for a Novel Endoplasmic Reticulum Complex Regulating Collagen Lysyl Hydroxylation

Melissa E. Heard, Roberta Besio, MaryAnn Weis, Jyoti Rai, David M. Hudson, Milena Dimori, Sarah M. Zimmerman, Jeffrey A. Kamykowski, William R. Hogue, Frances L. Swain, Marie S. Burdine, Samuel G. Mackintosh, Alan J. Tackett, Larry J. Suva, David R. Eyre, and Roy Morello

Sc65 (Synaptoneal Complex 65) is an endoplasmic reticulum protein that belongs to the Leprecan family which includes the prolyl 3-hydroxylases (P3H1, P3H2, P3H3) and cartilage associated protein (CRTAP). We and others have shown that mutations in both *CRTAP* and *LEPRE1* (encoding P3H1) cause recessive forms of Osteogenesis Imperfecta. Sc65 and CRTAP are both non-enzymatic proteins and share high homology suggesting Sc65 may also function in bone homeostasis. Utilizing a gene trap allele, we recently demonstrated that loss of Sc65 results in low bone mass. Here, a new global knockout mouse (Sc65-KO) derived by deleting exons 7 and 8 using homologous recombination is described. Mice null for Sc65 exhibit a similar bone loss phenotype as the previous model with decreased BV/TV and the loss of cortical and trabecular bone. To ascertain Sc65 function in bone, co-immunoprecipitation (co-IP) of Sc65 candidate interactors in mouse fibroblasts followed by mass spectrometry was performed. These experiments identified several fibrillar procollagen α-chains as likely substrates of Sc65 supporting the idea that Sc65 plays a role in collagen modification, similar to other Leprecans. At the biochemical level, mass spectrometry of type I collagen peptides showed severe under-hydroxylation at helical cross-linking sites K87 and K930/933 in collagen α1(I) and α2(I) chains both from bone and skin which are known LH1 preferred substrate residues but with no effect on sites of prolyl 3-hydroxylation. Direct co-IP assays showed Sc65 interaction with lysyl-hydroxylase 1 (LH1, Plod1), prolyl 3-hydroxylase 3 and cyclophilin B. Western blot revealed dramatic reduction of LH1 and P3H3 in primary osteoblasts and skin fibroblasts from *Sc65*-KO mice. Size exclusion chromatography confirmed that Sc65 and P3H3 form a stable complex in the ER that affects the activity of lysyl-hydroxylase 1 potentially through interactions with the enzyme and/or cyclophilin B. Further testing showed that *Sc65*-KO mice also have fragile skin with less tensile strength than control mice, consistent with a collagen cross-linking abnormality. Collectively, these results indicate that Sc65 is a novel adapter molecule that stabilizes a unique ER-resident complex that is essential for proper collagen lysyl-hydroxylation. Loss of Sc65 leads to complex instability and defective fibrillar collagen modifications which negatively impacts bone, skin and likely other connective tissues.

Poster #4

Platelet Alpha-Granules: One Flavor or Many?

Irina Pokrovskaya*, Jeffrey Kamykowski*, Emma McBride^^, Maria Aronova^^, Amith Rao^^, Richard Leapman^^, and Brian Storrie*

*Department of Physiology and Biophysics, University of Arkansas for Medical Sciences; ^^National Institute of Bioimaging and Bioengineering, National Institute of Health

Alpha-granules are the major protein storage granule within platelets and a wide range of soluble cargo proteins often of contradictory physiological function as well as membrane proteins such as p-selectin. Granule contents can be secreted through fusion with the plasma membrane through thin connections that we term, *pipes*. Recently published evidence from this laboratory suggests that pipes and likely controlled cargo deconden-sation filter cargo release (JTH, 2016). Whether that release is from a relatively homogenous or a decidedly heterogeneous population of a-granules remains an open question. Here we summarize work in progress using state of the art imaging approaches including serial block face (SBF) SEM, serial section immunogold labeling, and super resolution fluorescence microscopy that leads to the conclusion that alpha-granules in human fall predominantly into one major structural class.

Poster #5

Lipid Stress Alters Cell Distribution, Traffic, and Desensitization Properties of Melanocortin-4 Receptor, a GPCR Involved in Appetite Control

Kimberly A. Cooney, Brent M. Molden, and Giulia Baldini

Melanocortin-4 Receptor (MC4R) is a G-protein coupled receptor expressed in the brain hypothalamus where it regulates food intake and energy expenditure. Obesity is often associated with dislipidemia, a condition where the concentration of circulating Non-Esterified Fatty Acids (NEFAs), such as palmitic acid, is increased. We have modeled effects of lipid stress on MC4R function by exposing neuronal Neuro2A cells and immortalized hypothalamic mHypoE-42 neurons to elevated concentrations of palmitate within the physiological range. Lipid stress changes the cell shape of Neuro2A cells and inhibits internalization of transferrin receptor both in these cells and in the mHypoE-42 neurons. Superresolution fluorescence microscopy indicates that exposure to lipid stress inhibits transit of tagged HA-MC4R-GFP to early endosomes by halting exit of the receptor from clathrin-coated pits. In addition, lipid stress blunts desensitization of MC4R in response to the natural agonist alpha-MSH, and to the synthetic agonist Melanotan II (MTII). A single intraperitoneal injection of MTII decreases weight in obese male C57BL/6J mice exposed to high fat diet, but not in lean mice. These studies provide evidence that lipid stress may unexpectedly promote an increased response to anti-obesity treatments targeted to MC4R by changing traffic of the receptor.

Poster #10

Evidence that Endogenous G-quadruplex DNA Mediates Stress Granule Assembly in Response to Oxidative Stress

Alicia K. Byrd, Boris L. Zybailov, Leena Maddukuri, Jun Gao, John C. Marecki, Mihir Jaiswal, Matthew R. Bell, Wezley C. Griffin, Megan R. Reed, Shubeena Chib, Samuel G. Mackintosh, Angus M. MacNicol, Giulia Baldini, Robert L. Eoff, and Kevin D. Raney

DNA sequences that consist of appropriately spaced guanine repeats can readily fold into stable structures termed G-quadruplex DNA (G4DNA). These sequences occur in non-random sites throughout genomic DNA, such as the promoters of proto-oncogenes, but are especially concentrated in telomeres and human mitochondrial DNA. G4DNA is proposed to play specific roles in DNA metabolism including replication, gene expression and mitochondrial DNA metabolism. Human cancer and neurodegenerative diseases have been linked to misregulation of G4DNA or G4RNA. In order to understand the mechanisms and functions of G4DNA, the proteins that bind and/or unfold these structures need to be identified. We performed a quantitative proteomics analysis using G4DNA as bait and the top hit was the DHX36 RNA helicase, a protein known to tightly bind and unfold G4DNA. Surprisingly, the other major proteins discovered, such as TIA1 and YB1 can associate with mRNA within cytoplasmic stress granules. Stress granules are dynamic assemblies of protein and mRNA that regulate translation in response to the cellular environment. To support the proteomics data, fluorescence co-localization experiments were performed by introducing fluorescently labeled G4DNA into cells. Multiple stress granule proteins were found to co-localize in the cytoplasm with exogenous G4DNA introduced into cells by transfection. These results led us to speculate that endogenous G4DNA may appear in the cytoplasm as a result of stress, and then interact with stress granules. We demonstrate that sequences capable of forming G-quadruplex DNA appear at increasing levels in the cytoplasm after treatment with hydrogen peroxide. A quadruplex specific antibody, BG4, was used to examine the localization of G4DNA in cells in response to oxidative stress. Increasing amounts of fluorescent foci were observed in the cytoplasm as a function of time, consistent with the idea that G4DNA can emerge in the cytoplasm during stress. The fluorescent foci were found to co-localize with proteins found in stress granules such as TIA1 and G3BP. G4DNA is normally observed in the nucleus, but this evidence supports its appearance in the cytoplasm after oxidative stress. G4DNA is resistant to nuclease activity, allowing it to bind to proteins that are found in stress granules. Since one role of stress granules is to modulate translation, we propose that excised G4DNA can serve as a signaling molecule that modulates gene expression in response to oxidative stress. This new signaling pathway provides a mechanism whereby cells can rapidly respond to DNA damage caused by oxidative stress and exert a broad impact on gene expression. This new role for G-quadruplex DNA provides an additional molecular explanation for why such sequences are prevalent in the human genome.

Poster #9

FoxP3+ T-cells and PD-L1 Highlight Immune-Suppressive Profiles in Vulvar Squamous Cell Carcinoma

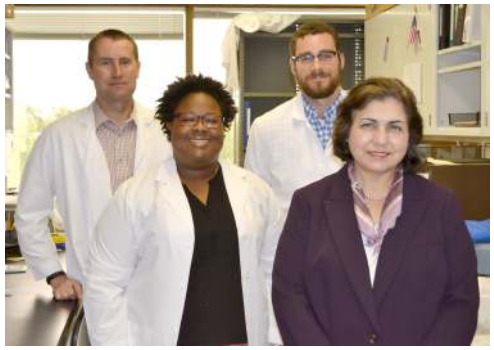
Emily Holthoff, Thomas Kelly PhD, Charles Quick MD, and Steven Post, PhD

Suppression of anti-tumor immune responses can be permissive for tumor proliferation and invasion. Increased populations of FoxP3+ T-regulatory cells and the production of programmed death-1 ligand (PD-L1) decrease CD8+ T-cell mediated immune response and are associated with worse outcomes in several cancers. Recent studies have shown that vulvar squamous cell carcinoma (vSCC) can exhibit two different stromal response patterns. One is a fibromyxoid (FMX) response displaying fibroblasts and immature collagen, and the other is a lymphoplasmacytic (LPC) response rich in T-lymphocytes and plasma cells. Tumors containing a FMX stromal response have worse clinical outcomes, including increased risk for nodal metastasis, when compared to tumors with a LPC stroma. One possible explanation for these poor outcomes is the absence of an immune response in tumors with an FMX stroma. The goal of this study was to determine whether FoxP3+ T-cells and PD-L1 play a role in immune modulation in vSCC with higher risk for nodal metastasis.

Our findings suggest that the high risk vSCC with FMX stroma utilize a method of immune suppression that is independent of FoxP3+ T-regulatory cells or PD-L1 expression. However, within the lower risk vSCC with LPC stroma, a subset of tumors has an immune-suppressive profile with an elevated FoxP3+ cell population and increased PD-L1 expression. These tumors are more likely than others with LPC stroma to exhibit nodal metastases and may be an important target for immunotherapy.

Poster #6

Cigarette Smoking Mediates the Membrane Trafficking of N-Glycan via Serotonin/Adrenalin Signaling Pathways of Platelets



Curtis Lee Lowery III, Clay Elliott, Coedy Hadden,
James D. Marsh, and Fusun Kilic

Cigarette smoking produces acute central nervous system-mediated activation of the sympathetic nervous system (SNS) that stimulates the secretion of serotonin (5-HT) and catecholamine into the blood at supraphysiological levels. This pathological incident is a major factor leading to acute blood clotting. In this study, we investigated the mechanism(s) in which 5-HT/catecholamine-mediated signaling predisposes platelets to aggregation. The MALDI-MS demonstrated that 15 min after smoking, the level of *N*-glycan on the platelet surface was 30% higher than before smoking, and this coincided with an increase in platelet aggregation. These findings were further evaluated in nonsmokers' platelets treated with supraphysiological levels of 5-HT/catecholamine with and without pharmacological interruptions of the 5-HT or catecholamine, β2-/α2-adrenergic receptors. The elevated intracellular free Ca level and transglutaminase activity link between smoking-activated platelet with phospholipase C and inositol-1,4,5-triphosphate pathways— the downstream effectors of the 5-HT/catecholamine signaling pathways. Our findings provide insight into the molecular mechanism by which cigarette smoking-associated elevation in plasma 5-HT and catecholamine levels predisposes platelets to thrombosis. We propose that the additive effect of 5-HT/catecholamine overcomes the b2-adrenergic receptor-associated inhibitory role of cAMP levels in the platelet aggregation process. Accordingly, a mixture of receptor antagonists and their intracellular signaling pathways may represent viable targets for novel antiplatelet agents.

Poster #7

C/EBP delta-deficient Mice Display Aberrant Inflammatory and Oxidative Stress Response that Promotes Radiation-induced Intestinal Injury and Barrier Disruption

Sudip Banerjee, Qiang Fu, Sumit K. Shah, Vaibhav D. Aher, Usha Ponnappan, Stepan B. Melnyk, Martin Hauer-Jensen and Snehalata A. Pawar

CCAAT enhancer binding protein delta (Cebpd, C/EBPδ) is a transcription factor implicated in the regulation of oxidative stress, DNA damage response and inflammation. We have previously shown that Cebpd-KO mice display lethality to radiation due to injury to the bone marrow and intestinal tissues. In this study, we investigated the underlying basis for radiation-induced intestinal injury, focusing on aberrant inflammatory and oxidative stress response. Methods: Intestine, liver and blood samples were harvested from Cebpd-WT and KO mice after exposure to 8.5 Gy. Gene expression of the inflammatory cytokines, chemokines and tight junction proteins in intestine tissue were measured by qRT-PCR. Plasma cytokine profiles were measured by ELISA. Intestinal barrier function of WT and KO mice was assessed by in vivo intestinal permeability assay. The levels of GSH and 3-NT were measured by HPLC-EC.
Results: Irradiated Cebpd-KO mice demonstrated significantly elevated expression of pro-inflammatory cytokines, chemokines and 3-NT and reduced levels of GSH, which correlated with increased expression of Claudin-2 and intestinal permeability. Conclusions: These results demonstrate that elevated inflammation and oxidative stress underlie IR-induced intestinal injury in KO mice.

Poster #8

Doxorubicin Inhibition of Lymphatic Function is Mediated by Ryanodine Receptors and Prevented by Dantrolene

Amanda J. Stolarz, Asif R. Pathan, Rachel Versluis, Terry W. Fletcher, Joseph R. Stimers, Mustafa Sarimollaoglu, Ekaterina Galanzha, Vladimir Zharov, and Nancy J. Rusch

Doxorubicin (DOX) is a risk factor for lymphedema in breast cancer patients after surgery and/or radiation. The mechanisms by which DOX contributes to lymphedema are unclear. We hypothesized that distinct from its cytotoxic actions, DOX directly inhibits lymph vessel (LV) spontaneous contractions and lymph flow by opening ryanodine receptors (RyRs) and disrupting the finely tuned calcium signaling pathways critical to LV contractions. Isolated rat mesenteric LVs were cannulated, pressurized (4-5 mm Hg) and equilibrated in physiological salt solution (37°C) until contractions were stable. Edge-detection software recorded changes in external diameter. Adding DOX (0.5 to 20 µM) to the bath solution progressively diminished LV contractions, culminating in reductions in amplitude (~70%), area under the curve (~62%), resting diameter (~24%), and frequency (~30%) (n=12). Incubation with dantrolene (10 µM), a clinically available RyR1 inhibitor, significantly prevented DOX inhibitions to <15% reduction from baseline (n=6). High-speed, intravital optical imaging of rat mesenteric LVs *in vivo* revealed that topically applied DOX reduced positive volumetric lymph flow (IC_{50}= 1.52 µM; n=6). Thus, DOX may contribute to lymphedema by opening RyR1s and suppressing LV contractions and lymph flow, and dantrolene may represent an unrecognized therapeutic option to prevent DOX inhibition of lymphatic function.