Intracellular trafficking of a pH-responsive drug metal complex

Azadeh Kheirolomoom[a,1,*], Elizabeth S. Ingham[a,1], Joel Commisso[b], Neveen Abushaban[a], and Katherine W. Ferrara[a,*]

[a]University of California, Davis, Department of Biomedical Engineering, 451 East Health Sciences Drive, Davis, CA 95616, USA
[b]University of California, Davis, Interdisciplinary Center for Plasma Mass Spectrometry, Davis, CA 95616, USA

Azadeh Kheirolomoom, akheirolomoom@ucdavis.edu
Elizabeth S. Ingham, esingham@ucdavis.edu
Joel Commisso, jcommisso@ucdavis.edu
Neveen Abushaban, nevaban@ucdavis.edu
Katherine W. Ferrara, kwferrara@ucdavis.edu

Running title: Doxorubicin in copper-doxorubicin complex traffics to nucleus

Keywords: Doxorubicin, Liposome, Intracellular trafficking, Mild hyperthermia

[1]These two authors contributed equally to this work.

*Corresponding authors: Azadeh Kheirolomoom and Katherine W. Ferrara, One Shields Avenue, Department of Biomedical Engineering, UC Davis, Davis CA 95616, USA, (530)754-9436, FAX (530)754-5739, akheirolomoom@ucdavis.edu and kwferrara@ucdavis.edu

Supplementary Information

Materials and methods

Materials

1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (MPPC), and 1,2 distearoyl-sn-glycero-3-phosphoethanolamine-N-Methoxy polyethyleneglycol-2000 (DSPE-PEG2k) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Copper (II) gluconate, triethanolamine (TEA), ammonium sulfate, and doxorubicin hydrochloride were from Sigma (St. Louis, MO). LysoTracker® Blue DND-22 was from Invitrogen (Carlsbad, CA).

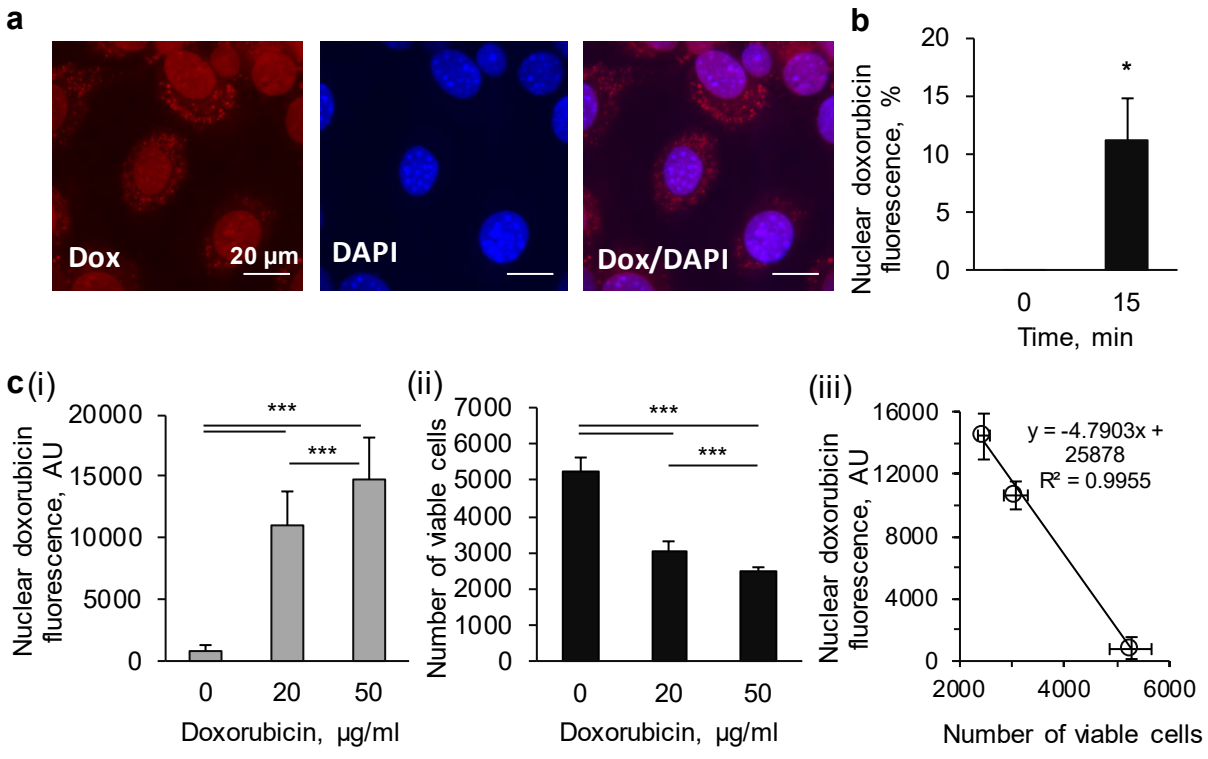
Liposome preparation

The dried lipid was hydrated in 0.3 ml of either 250 mM ammonium sulfate (AS) or 100 mM copper (II) gluconate including triethanolamine at various concentrations of 270 mM (pH 7.4), 540 mM (pH 8.4), 810 mM (pH 8.8). The multi-lamellar lipid solution at a final concentration of 50 mg/ml was extruded above the phase transition temperature of the lipid mixture through a polycarbonate membrane with a pore diameter of 100 nm. To induce a salt gradient across the liposomal membrane, ammonium sulfate-loaded or copper/TEA-loaded liposomes were

separated from non-encapsulated ammonium sulfate or copper/TEA by passing the extruded liposomal suspension through a spin column of Sephadex G-75 (5 x 1 cm, GE Healthcare, Biosciences, Piscataway, NJ) equilibrated with 20 mM HEPES/150 mM sodium chloride and saline (0.9% sodium chloride), respectively. The liposomal diameters were ~100 nm (105 nm ± 15 nm) as measured using a NICOMP™ 380 ZLS submicron particle analyzer (Particle Sizing System Inc., Santa Barbara, CA). Lipid concentration was measured using the Phospholipids C assay kit (Wako Chemicals USA, Richmond, VA) according to manufacturer's instructions.

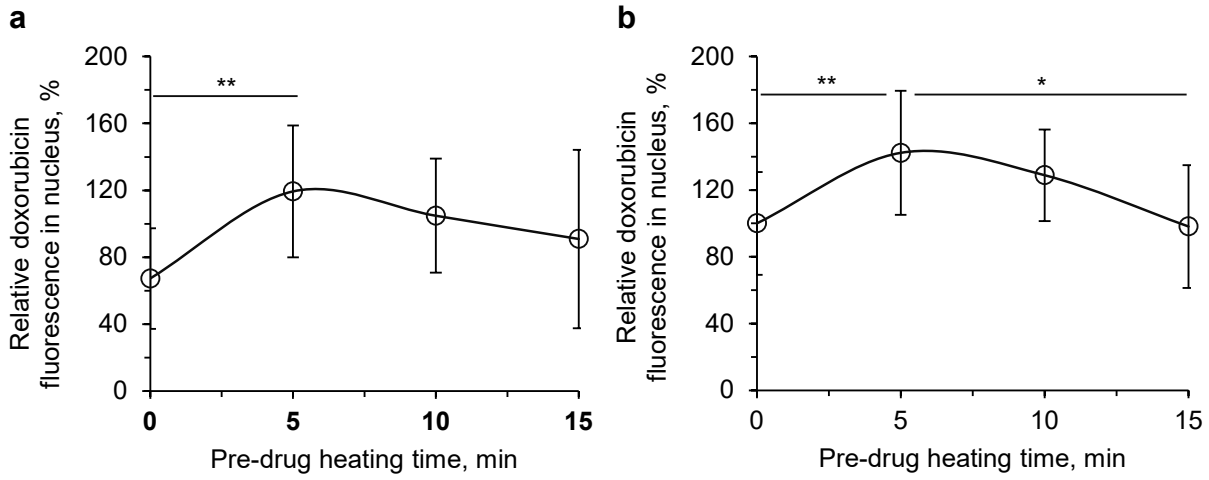
Cell culture

The *neu* deletion (NDL) and MET-1 metastatic murine mammary carcinoma cell lines were cultured in DMEM (Dulbecco modified Eagle medium) high-glucose complete medium (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum,1% penicillin–streptomycin, 0.584 g/l L-glutamine and 0.110 g/l sodium pyruvate. Human malignant melanoma (H1 Melanoma) cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, four times the prescribed concentration of non-essential amino acids, 2% L-Glutamine and 1% penicillin–streptomycin [1]. Human umbilical vein endothelial cells (HUVECs) were cultured in endothelial growth medium (EGM, Lonza) supplemented with 0.1% (v/v) human endothelial growth factor (hEGF), 0.1% (v/v) hydrocortisone, 0.1% (v/v) GA-1000, 0.4% (v/v) bovine brain extract (BBE) and 10% (v/v) fetal bovine serum (FBS), all from Lonza, Inc (Walkersville, MD). All cells were maintained at 37°C in a humidified 5% CO2 incubator and growth medium was exchanged twice a week.



Supplementary Figure S1. Cellular uptake and intracellular distribution of free Dox. a) Fluorescence images of NDL cells incubated with 20 µg free Dox (Free Dox) for 30 min at 4°C,

washed and incubated with media for 24 h at 37°C and stained with DAPI to confirm localization of Dox accumulation in the nucleus. Red, blue and purple colors indicate Dox fluorescence, DAPI fluorescence and co-localization of Dox and DAPI, respectively. b) Dox fluorescence intensity quantified in the nucleus of NDL cells post 30 min incubation with 20 μg free Dox at 4°C, washed, and compared to that quantified after 15 min incubation at 37°C. c) Nuclear fluorescence intensity of Dox (i) and *in vitro* viability of NDL cells (ii) as a function of Free Dox concentration in media at 24 h. (iii) Correlation between nuclear Dox fluorescence and number of viable cells. Statistical analyses were performed using Student's *t*-test (b) and one-way ANOVA followed by a Tukey Post Hoc test (c). Scale bars, 20 μm. **p* < 0.05, ****p* < 0.001. AU stands for arbitrary units.



Supplementary Figure S2. Timing of heat and drug exposure in NDL cells. Dox fluorescence at 5 h (a) and 24 h (b) after cellular heat treatment, given as a function of incubation time of NDL cells at 42°C prior to the addition of free Dox . Cells were placed on a heat block at 42°C for 0, 5, 10, and 15 min and reached room temperature for 5 min prior to incubation at 4°C for 30 min. Cells were then incubated with free Dox at 20 μg/ml at 4°C for an additional 30 min, rinsed twice with cold media and incubated at 37°C for 5 and 24 h. Statistical analyses were performed using one-way ANOVA followed by a Tukey Post Hoc test. **p* < 0.05, ***p* < 0.01.