Increased pressure alters plasma membrane dynamics and renders acute myeloid leukemia cells resistant to daunorubicin

Victor Sanjit Nirmalanandhan,[1] Rose Hurren,[1] William D. Cameron,[2] Marcela Gronda,[1] Aisha Shamas-Din,[1] Lidan You,[2] Mark D. Minden,[1] Jonathan V. Rocheleau,[2,3] and Aaron D Schimmer[1]

[1]Princess Margaret Cancer Centre, University Health Network, Toronto, ON; [2]Institute of Biomaterials and Biomedical Engineering, University of Toronto; [3]and Toronto General Research Institute, University Health Network, Toronto, ON, Canada

Correspondence: aaron.schimmer@utoronto.ca
doi:10.3324/haematol.2015.129866

SUPPLEMTAL METHODS

Two-photon microscopy and image analysis:

Cells were stained with 5 µM of Laurdan (Sigma Aldrich, St. Louis, MO) dye for 30 minutes, transferred to 35 mm glass bottom µ-Dish (ibidi, Martinsried, Germany) coated with cell-tak (BD BioScience, Bedford, MA) and centrifuged at 700 rpm for 2 minutes. The media was aspirated and 1 ml of imaging buffer (125 mM NaCl, 5.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM HEPES, pH = 7.4) was added to each dish. Imaging was performed on a Zeiss LSM710 confocal microscope (Zeiss) equipped with a Chameleon two-photon (2P) laser (Coherent) and a heating chamber to maintain the temperature at 37ºC. Images were collected at atmospheric pressure (Atm) with a 63 × oil immersion objective (NA=1.4) using a dwell time of 25.21 µs, image size of 512 × 512 and a zoom factor of 2.0 (pixel size = 130 × 130 nm). To minimize the reversal of pressure induced changes in membrane dynamics, the cells that were exposed to high pressure were maintained in the pressure chamber until imaging and imaged within 10 minutes of removing from the pressure chamber**.** Laurdan fluorescence was excited at 770 nm and collected simultaneously at emission bandwiths 400-460 nm and 470-530 nm. For both channels, detector gain was set to 900, digital offset was set to 0, and digital gain was set to 1.

Laurdan generalized polarization (GP) was calculated using a custom imageJ plugin. Briefly, background subtraction was performed using a manual selection of a non-fluorescent region of the image. A median filter (radius=2) was then applied to the image and a manual selection of a threshold was used on the 400-460 nm channel to facilitate membrane selection. This threshold was used to create a mask that was applied equally to the two channels, excluding pixels above (saturation) or below the threshold for future measurements. Membranes were then traced manually and the average intensity values of each channel were calculated and exported to a text file. GP values were then calculated using the equation:

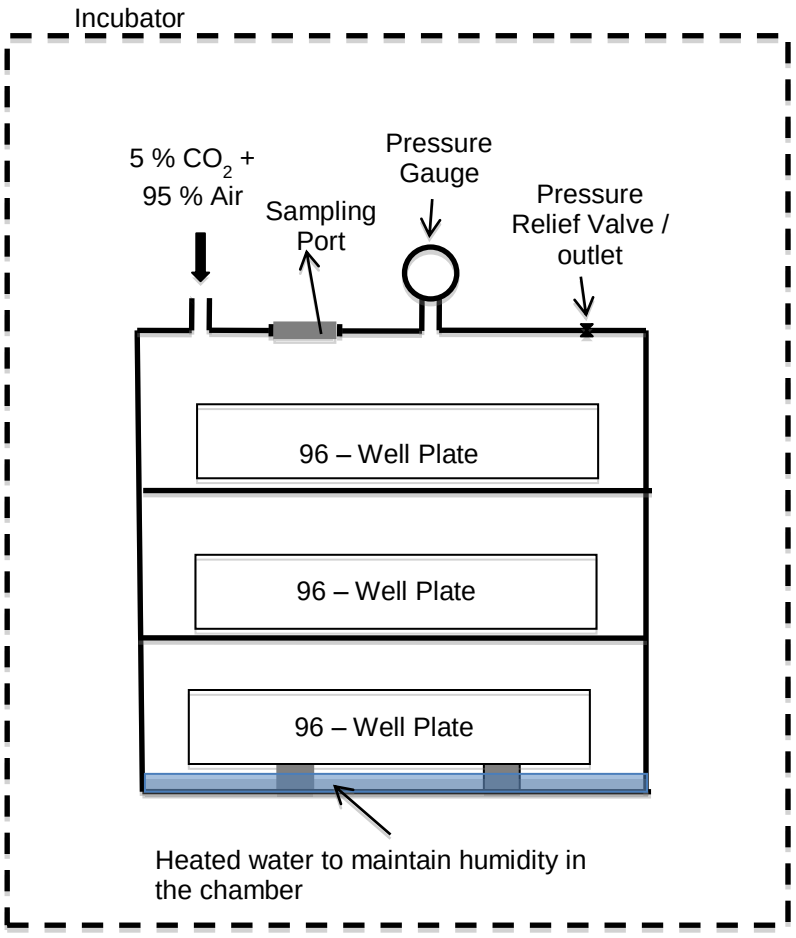
$$GP = \frac{I_{400-460\ nm} - G \cdot I_{470-530\ nm}}{I_{400-460\ nm} + G \cdot I_{470-530\ nm}}$$

where I represents the exported measured intensities and G represents the correction factor calculated through two variables: a known GP value for laurdan in DMSO at 22°C and the GP value of the laurdan stock solution (500 mM) determined in each experiment. The use of the correction factor was used to control for differences in measurements across the two channels and was re-measured for each experiment.

Supplemental Table 1: Characteristics of patients used in the study.

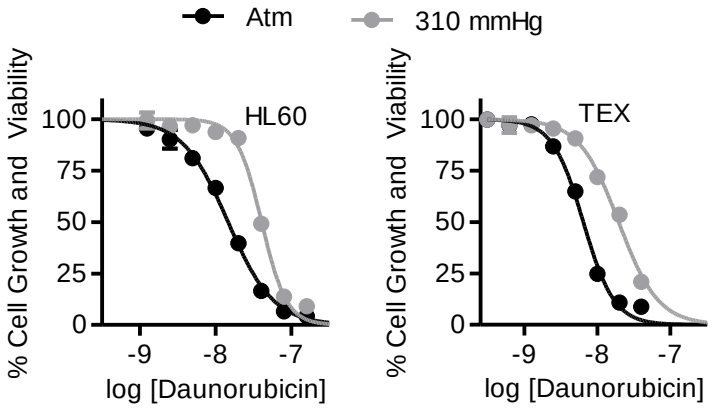
Patient ID	Diagnosis (WHO)	Gender	Age	Cytogenetics	Molecular
1	AML	Male	75	48,XY,+9,+13[4]/46,XY[16]	JAK2 V617F neg
2	AML, M5b	Female	56	46,XX [20]	NPM1 pos, FLT3-ITD pos, FLT3-TKD neg
3	AML, M1	Female	76	inconclusive	NPM1 pos, FLT3-ITD pos, FLT3-TKD neg
4	T-ALL	Female	51	47,XX,+8,del(9)(p13),t(11;14)(p13;q11.2~14)[8]/46,XX[12]	not done
5	t-AML, post MDS	Female	78	not done	not done

SUPPLEMENTAL FIGURES



Supplemental Figure 1: Pressure Chamber.

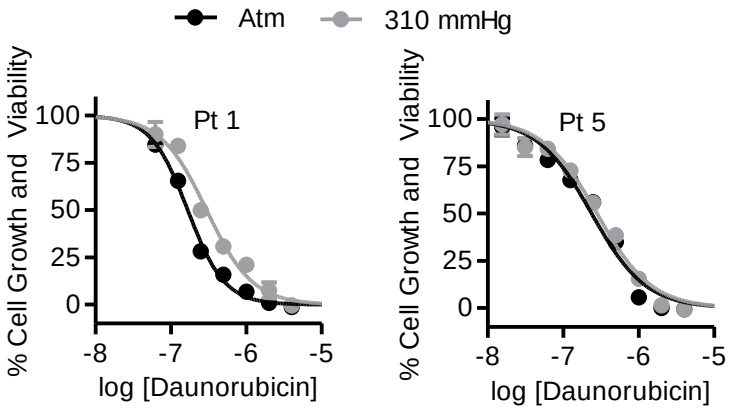
The schematic of the 5" x 7" x 8" (W x D x H) plexiglass chamber custom machined with 3 removable perforated shelves to accommodate 3 standard multi-well plates is shown. The pressure chamber fits inside a standard CO_2 incubator. The chamber is pressurized by continuously circulating a 5% CO_2 and 95% air mixture through the inlet and outlet at high pressure. The pressure gauge monitors the pressure in the chamber and any excess pressure is released through the pressure relief valve. The chamber also contains a water reservoir at the bottom to maintain a humid atmosphere. The air in the chamber and/or the media in the cell plate can be sampled through the sampling port, if required. Schematic is not drawn to scale.



	IC_{50}	
	Atm	**310 mmHg**
HL60	14.6 ± 1.2 nM	40.5 ± 1.3 nM
TEX	6.4 ± 0.1 nM	19.5 ± 0.9 nM

Supplemental Figure 2: Effects of increased pressure on sensitivity to daunorubicin in AML cell lines.

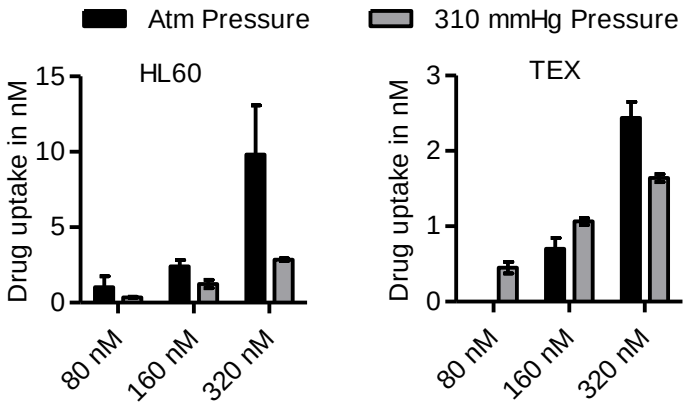
HL60 and TEX cells were cultured at Atm and 310 mmHg above Atm for 3 days, and then treated with increasing concentrations of daunorubicin at indicated pressures for an additional 3 days. Cell viability was determined by Celltiter Fluor assay and IC_{50}'s were calculated using a variable slope equation in GraphPad Prism for each experiment. Each point is a mean ± SEM from three independent experiments.



	IC_{50}	
	Atm	**310 mmHg**
Patient 1	167 ± 2 nM	299 ± 30 nM
Patient 5	238 ± 12 nM	279 ± 22 nM

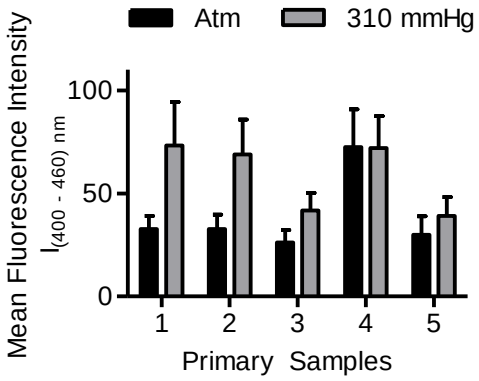
Supplemental Figure 3: Effects of increase pressure on sensitivity to daunorubicin in primary patient samples.

Primary patient cells were cultured at Atm and 310 mmHg above Atm for 3 days, and then treated with increasing concentrations of daunorubicin at indicated pressures for an additional 3 days. Cell viability was determined by Celltiter Fluor assay and IC_{50}'s were calculated using a variable slope equation in GraphPad Prism for each experiment. Each point is a mean ± SEM from three independent experiments.



Supplemental Figure 4: Effects of increase pressure on the uptake of daunorubicin.

TEX and HL60 cells were cultured at 37°C at Atm and 310 mmHg above Atm for 3 days and then treated with 8 nM of [^{3}H] daunorubicin for 3 hours at the same pressure levels. Following treatment, intracellular levels of radiolabeled daunorubicin were measured using a scintillation counter. The counts per minute (CPM) of radioactivity from Figure 2A were converted to concentration of daunorubicin using a standard curve of serially diluted [^{3}H] daunorubicin.



Supplemental Figure 5. Mean fluorescence intensity of laurdan from 400-460 nm channel.

Primary AML patient cells (1 x 10^6 cells) were cultured at 37°C at Atm and 310 mmHg above Atm for 3 days, and then stained with 10 µM of the lipophilic probe, laurdan for 30 minutes. The emission state of laurdan was assessed using two-photon microscopy at 37°C, Atm and within 10 minutes of removing from the pressure chamber. The fluorescence spectrum of laurdan is sensitive to the physical state of membrane phospholipids; therefore, when excited, laurdan emits at 440 nm when the membrane is in a gel-like state and at 490 nm when the membrane is in a liquid-crystalline phase. Data represent the mean ± SD of mean intensity values from 400-460 nm channel from 30-60 cells.