Supporting Information

Probing Arsenic Trioxide (ATO) Treated Leukemia Cell Elasticities Using Atomic Force Microscopy

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Materials and Methods

Drug Treatment

For the viability and AFM experiments on NB4 cells, 10 mL cell solution of 10^5 cells/mL was aliquoted in multiple 25 cm^2 cell culture flasks (Fisher Scientific, PA, USA). Successively, each flask of NB4 cells was treated with a single dose of ATO (Sigma-Aldrich, WI, USA) solution prepared in 1X Gibco® PBS of pH 7.4, without calcium or magnesium (Life Technologies, NY, USA). This PBS is used in all experiments, unless otherwise stated.

Viability experiments were repeated 6 times using ATO concentrations of 0, 5, 10, 20 and 30 µM. Following viability testing, caspase assays and AFM experiments were performed over 48 h using a 30 µM ATO treatment only.

Preparation of Micro-wells

An SU8-10 photoresist solution was used to create micro-well arrays to physically confine APL cells, details were previously published. Briefly, the SU8-10 film was fabricated on a clean coverslip and wells were designed using a photomask (HTA Photomask, CA, USA) patterned by an array of 20 μm circles. Well depth was controlled between 7 and 8 μm and confirmed using the cyberSCAN CT 100 high resolution non-contact profilometer (Cyber Technologies, Germany). SU8-10 substrate was inspected to avoid using any cracked films.[1]

AFM Measurements and Data Processing

Both the SU8-10 micro-well substrate and AFM liquid cell were thoroughly rinsed using 70% ethanol first and then 18 MΩ·cm MilliQ H_2O. The SU8-10 substrate was then placed at the bottom of a closed liquid cell (JPK Instruments CoverslipHolder), which was successively filled with 800 μL of DMEM with 1X penicillin before placing it into a vacuum chamber. After vacuuming for 30 minutes, no air bubbles were visible. It should be noted that no fetal bovine serum was used due to its viscosity which can cause foaming within vacuuming in chamber. The DMEM and penicillin solution were systematically replaced by 200 μL of warm culturing media at 37 °C, followed by the addition of 100 μL of cell solution in the liquid cell. The whole sample was placed on the AFM stage for 10 minutes, to ensure the NB4 cells settled inside the micro-wells.

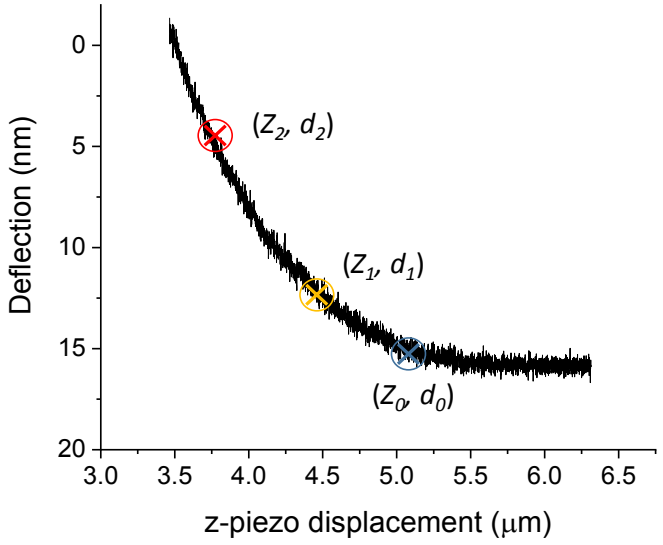
Fitting model

Fifteen force sets (49 curves per set) were batch analyzed using a self-developed code implemented in IGOR Pro 6 (Wavemetrics, Portland, OR, USA) to quickly process massive amounts of force curves. For each curve, the Young's modulus was extracted using the spherical equation and model. Fitting evaluations were done to define specific criteria such as the contact

point location and indentation range that minimize the fitting errors. Force curves were thus fit using a spherical probe and model in order to calculate the Young's modulus (E) derived from the cantilever deflection (d) as a function of the ramp of the z-piezo (Z) and the cantilever's spring constant (k):

$$Z = Z_0 + (d - d_0) + \left[\frac{3k(d - d_0)(1 - \nu^2)}{4E\sqrt{R}}\right]^a \quad . \tag{S1}$$

Here in this spherical model, a=2/3. ν (0.5) and R (10 μm) are Poisson's value and tip radius, respectively. Each data point, i, of a deflection-displacement curve can be defined as (Z_i, d_i) and the contact point being labeled by the index 0 (Z_0, d_0). The deflection-ramp curves were fit using the least-square method.



Scheme S1. Reference points (Z_1, d_1) and (Z_2, d_2) located between 0% and 100% of the deflection, used to determine the contact point (Z_0, d_0) on an experimentally captured deflection-displacement (approaching) curve.

It is important to mention that as the fitting model has certain limitations, we were careful to use a large 20 μm diameter colloidal tip to obtain a good representation of the cell's heterogeneous surface. It was also important to use a small indentation force to gently indent the cell and avoid any substrate effect.

Microplate Fluorescence Readings

A FLUOstar Omega microplate reader (BMG Labtech, ON, Canada) was used for all fluorescence experiments, together with the LIVE/DEAD® Viability/Cytotoxicity Kit for Mammalian Cells (Life Technologies, NY, USA), and EnzChek® Caspase-3 Assay Kit #2 (Molecular Probes™, OR, USA). These fluorescence viability assays do not provide information about the absolute total cell population but only about the relative live and dead cell populations or caspase activity from one sample to another. As these measurements are population dependent, a batch of cells was aliquoted before treatment and measurements. Relative live/dead cell population as well as caspase activity were measured and calculated by comparing them to the largest and smallest fluorescence intensity readings from the adequate filters. More details can be found below.

Trypan Blue Staining and LDH Assay

NB4 cells were treated with 0, 5, 10, 20 and 30 μM ATO in PBS and the amount of live and dead cells were monitored at 0, 6, 24, and 48 h with the Trypan Blue assay. Cell solutions were diluted 1:1 with the Trypan Blue dye and the number of living cells (unstained cells) and dead

cells (stained cells) were optically quantified using LUNA™ Automated Cell Counter (Logos Biosystems, Inc., VA, USA). The average viability, the percentage of living cells to total cells, was measured at two different locations on the cell counting slides, on two separate slides for a total of four measurements for each ATO concentration and time interval. These measurements provided a preliminary assessment of the drug effects on NB4 cells within 48 h. (A)(A)

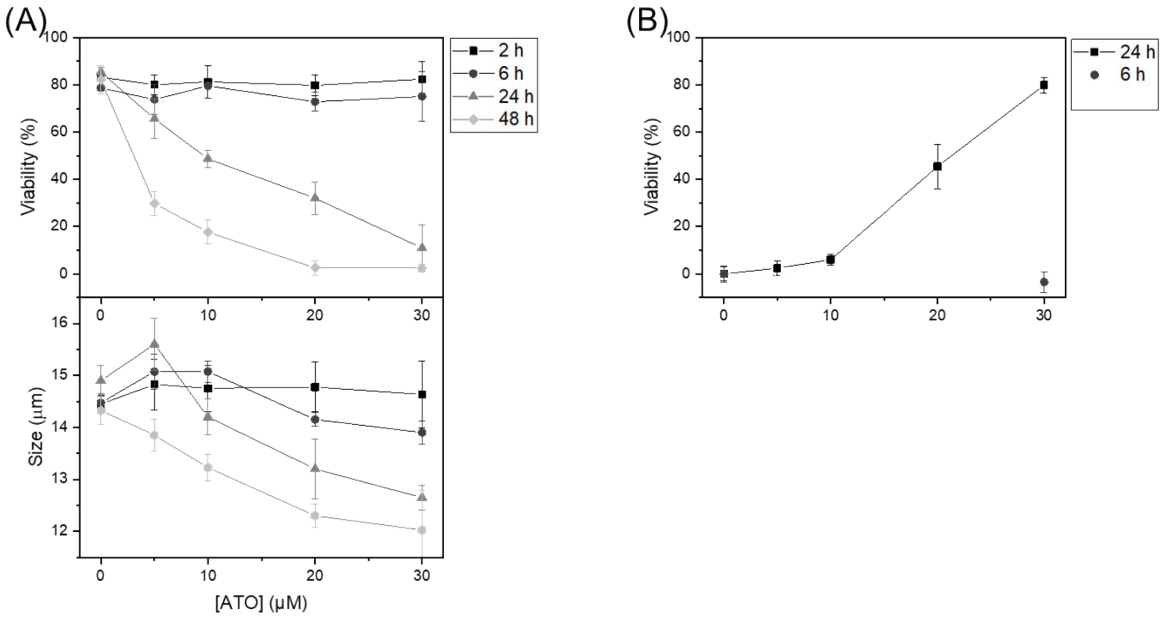


Figure S1. (A) Viability of NB4 cells exposed to different concentrations of ATO after 2, 6, 24 and 48 h by the trypan blue assay, with the cell sizes determined; (B) LDH released by cells undergoing apoptosis or necrosis after 24 h ATO treatment.

The Lactate dehydrogenase (LDH)-Cytotoxicity Assay Kit II (Abcam, CBG, UK) was carried out in clear 96-well, flat bottom plates (Fisher Scientific, ON, Canada). NB4 cells were treated with 5, 10, 20 and 30 μM ATO in PBS for 6 and 24 h post-treatment, where the assay reagents were added to all wells for their respective assays. The cells were then allowed to incubate for 4

h with the assay reagent in the incubator. For the LDH assay, after 4 h, the optical density of the cell samples and controls was immediately measured at 450 nm.

The trypan blue assay measures the viability of a cell sample based on the number of cells with an intact membrane compared to the total cell count. This assay is limited by the sensitivity and threshold of the instrument to distinguish between stained and unstained cells. As such, employing another assay to measure cell membrane integrity can bring more strength to the trypan blue assay results. The LDH assay was employed to accomplish this. The LDH and trypan blue assays have been previously demonstrated to consistently provide similar viability results under proper experimental conditions.[2] This assay measures the amount of LDH released by cells undergoing apoptosis or necrosis with a compromised or permeable membrane as released LDH reduces the assay reagent to a coloured formazan.[3] The amount of LDH released by the treated cell sample may then be compared to a negative control (untreated, living cells) and a positive control (completely lysed cells) to obtain a percent increase of LDH release.

WST-8, MST and MTT Viability Assay Tests

For cytotoxicity testing, the WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, VistaLab Technologies, Brewster, USA) and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma Aldrich, WI, USA) assays were used. The WST-8 assay, after 4 h, the optical density of the cell samples was immediately measured at 450 nm. For the MTT assay, the cells were centrifuged at 800 rpm for 5 minutes, the supernatant media was removed and replaced with DMSO. The MTT crystals were allowed to dissolve in the DMSO for 30 minutes at room temperature before the optical density of each

sample was read at 490 nm. Wells containing only media were taken as a negative control and wells treated with only PBS were taken as the positive control.

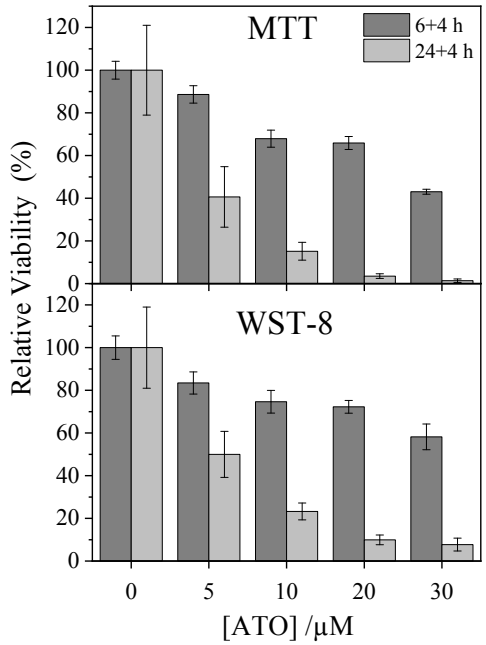


Figure S2: Viability of NB4 cells post exposure to 5, 10, 20 and 30 μM ATO for 6 and 24 h.

LIVE/DEAD® Cytotoxicity Test

LIVE/DEAD® Viability/Cytotoxicity Kit for Mammalian Cells (Life Technologies, NY, USA) was applied to NB4 cells, which were treated with 30 μM ATO in PBS for 2, 4, 6, 12, 24, 36 and 48 h. Cells were pelleted and washed once with PBS before being resuspended in PBS. A 1:1 ratio of cell solution to labelling assay reagent (prepared as per kit protocol) were combined. The labelling assay contained calcein AM and ethidium-1 to label the live and dead cells respectively. The cell solutions were incubated with the assay reagent in the incubator for 25 minutes, and their fluorescent intensities from 465 to 556 nm and 540 to 648 nm were recorded on the microplate reader.

LIVE/DEAD Assay protocol

The LIVE/DEAD® Viability/Cytotoxicity Kit contains both ethidium-1 (EthD-1) and calcein AM stock solutions. 15 µL of the 2 mM EthD-1 stock solution was added to 10 mL of PBS. Subsequent to mixing using a vortexer (VWR Scientific, PA, USA), 4 µL of the 4 mM calcein AM stock solution was added to the PBS and EthD-1 solution, and further vortexed to finalize the labelling solution.

Cells were treated with 30 µM ATO for 2, 4, 6, 12, 24, 36 and 48 h. Cell solutions were centrifuged at 800 rpm for 5 min and supernatant was aspirated. Cells were then washed in warm PBS, and centrifugation and supernatant removal was repeated, before resuspending the NB4 cells in warm PBS.

100 µL of the cell solution in PBS was placed in each well of the 96-well plate (Fisher Scientific, ON, Canada). An equal volume of 100 µL labelling solution was then added to each well. The 96-well plate was kept in the 37 °C incubator for 25 min before any reading experiments. The fluorescence of each sample was measured on the microplate reader. Plates were read using the recommended standard FITC filter (465 to 556 nm) to detect living cells. A Texas Red filter (540 to 648 nm) was chosen for the detection of dead cells in order to detect EthD-1 fluorescence emitted in the presence of DNA.

For each assessed sample, fluorescence intensity readings from 48 respective wells were collected using the specified filters. The adjustable gain was set at 10% in order to effectively record larger intensity readings at early (large amount of live cells) and later (large amount of dead cells) stages of the treatments. The background intensity value was averaged from 16 wells containing 100 µL of PBS mixed with 100 µL of labelling solution. Live and dead cell

populations were assessed simultaneously for each microplate within the microplate reader. 48 wells containing 100 µL of cell solution each were assessed for the monitored time point.

These fluorescence intensity readings involved not only more measurement values than when performing Trypan Blue staining assays, but also larger sampling offering statistically meaningful data. The smallest fluorescence intensity reading for calcein AM with an emission wavelength of 535 nm was associated to a relative live cell population of 0%, meanwhile the largest reading recorded was associated to a population of live cells only (100%). Same rules applied in the calculation of relative dead cell populations by recording the emission wavelength of EthD-1 at 620 nm. The positive control (PBS treated cell solution) was used to record the average background measured by the instrument, which was subtracted from each measurement value. Equations S2 and S3 for live (535 nm filter) and dead (620 nm filter) cell population were the following:

$$\%\ live\ cells = \frac{\left(I_{sample}^{535} - I_{bgd}^{535}\right) - \left(I_{min}^{535} - I_{bgd}^{535}\right)}{\left(I_{max}^{535} - I_{bgd}^{535}\right) - \left(I_{min}^{535} - I_{bgd}^{535}\right)} \times 100 \tag{S2}$$

$$\%\ dead\ cells = \frac{\left(I_{sample}^{620} - I_{bgd}^{620}\right) - \left(I_{min}^{620} - I_{bgd}^{620}\right)}{\left(I_{max}^{620} - I_{bgd}^{620}\right) - \left(I_{min}^{620} - I_{bgd}^{620}\right)} \times 100 \tag{S3}$$

where I represents the measured fluorescence intensity reading and superscripts 535 and 620 represent emission wavelength for the calcein AM (live/green, 535 nm) and EthD-1 (dead/red, 620 nm). Additional indexes in subscript ($sample$, max, min and bgd) characterize the readings for the sample, maximum, minimum and background intensity readings in that order.

Live/Dead justification

When taking a closer look at the effects of the 30 μM ATO concentration, the data distribution appears much narrower compared to the other ATO treatment concentrations. This result indicates that the ATO drug has significant effects on most of the NB4 cells when using a concentration of 30 μM. At early and later stages, the dead and live cell populations are also observed to be accurately measured, creating reliable reference points for the subsequent AFM experiments.

Another particularity when using 30 μM is the presence of a timeframe within which drastic increase in dead cells and decrease in live ones can be observed. Those changes are found between 6 and 24 h after treatment. Within that timeframe, the cells were greatly affected by the treatment.

EnzChek® Caspase-3 Assay Kit #2

A second microplate fluorescence reading experiment was performed using an EnzChek® Caspase-3 Assay Kit #2 (Molecular Probes™, OR, USA) to measure an earlier apoptosis stage. The kit contains rhodamine 110 bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide) (Z-DEVD–R110) substrate which involves peak excitation and emission wavelengths at 496 nm and 520 nm, respectively. Cells were treated with 30 μM ATO in PBS for 2, 4, 6, 12, 24, 36 and 48 h. Cells were pelleted and washed once with PBS before being suspended in cell lysis buffer solution and underwent a freeze thaw cycle. The lysed cells were centrifuged and combined in a micro-well plate in a 1:1 ratio with a 2X working solution containing rhodamine 110 bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide) (Z-DEVD–R110). Upon cleavage by active caspase-3 or caspase-7, the Z-DEVD-R110 generates a highly fluorescent product (rhodamine) that can be measured. Fluorescence emitting from the rhodamine is thus

proportional to the amount of caspase activity within a sample. At the desired post-treatment time, cells were centrifuged at 1000 rpm for 6 min and washed in PBS. After repeating centrifuging and repeated supernatant removal, the cells were resuspended in 350 µL of 1X cell lysis buffer solution and subjected to a freeze-thaw cycle by placing them in an ice-ethanol bath for 5 minutes. After centrifuging the lysed cells, 7 wells were filled with 50 µL of cell lysate and an additional 50 µL of 2X working solution. The micro-well plate was then incubated for 30 minutes at 37 °C before its fluorescence was measured. A blank 1X cell lysis solution was used to measure the background of the fluorescence readings.

Caspase activity was measured similarly to the relative live and dead cell populations when using the LIVE/DEAD® Viability/Cytotoxicity Kit. The smallest fluorescence intensity reading was associated to a relative caspase activity of 0%, meanwhile the largest reading recorded was associated to a relative caspase activity of 100%.

$$\%\ caspase\ activity = \frac{\left(I_{sample}^{535} - I_{bgd}^{535}\right) - \left(I_{min}^{535} - I_{bgd}^{535}\right)}{\left(I_{max}^{535} - I_{bgd}^{535}\right) - \left(I_{min}^{535} - I_{bgd}^{535}\right)} \times 100 \tag{S4}$$

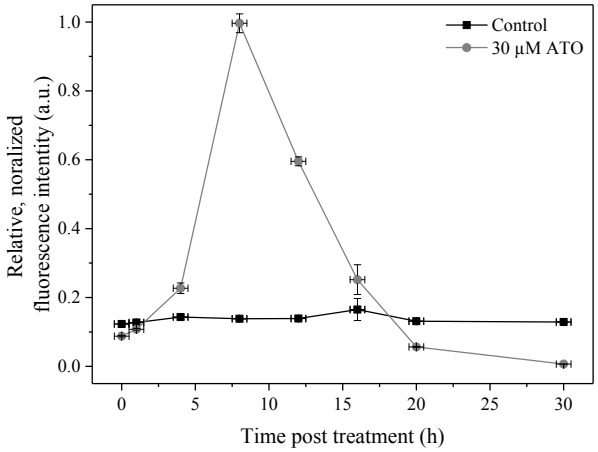


Figure S3. Microplate fluorescence intensity readings using an EnzChek Caspase-3 Assay kit with Z-DEVD-R110 to label caspase activity within an untreated cell sample as well as for cells treated using 30 µM ATO, from 0 to 30 h after treatment. Each data point represents the relative

caspase activity (%) for 8 well measurements taken within a 96-well plate. Y error bars represent the standard deviation of the caspase activity measurements and X error bars represent ± 0.5 h uncertainty for each time post-treatment.

The background intensity value was averaged from 7 wells containing 50 µL of blank 1X cell lysis solution mixed with 50 µL of 2X working solution within each measured plate. In the case of treated and untreated cell solutions, 7 wells were measured for each sample at 0, 2, 4, 8, 12, 16, 20 and 30 h post-treatment. Fluorescence reading was carried out 5 times for each microplate in order to include instrumental error.

References

(1) Niu, N.; Wang, L. In Vitro Human Cell Line Models to Predict Clinical Response to Anticancer Drugs. *Pharmacogenomics* **2015**, *16*, 273–285.

(2) Zhang, G.; Yang, Y. M.; Meng, W. T.; Zhou, J. Apoptosis of NB4 Cells Induced by Tanshinone II A Combined with Arsenic Trioxide. *Sichuan Daxue Xuebao, Yixueban* **2010**, *41*, 57–61.

(3) Luo, W. D.; Ren, C. M.; Zhu, M.; Chen, B. G.; Li, B. L.; Dai, M. Z.; Guo, Q. Y. Study on NB4 Cell Apoptosis Induced by Trichosanthin. *Zhongguo Shiyan Xueyexue Zazhi* **2005**, *13*, 278–281.