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**Taxol reduces synergistic, mechanobiological invasiveness of metastatic cells**

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**Abstract**

**Introduction**: Paclitaxel (Taxol) is chemotherapy drug widely used to treat different types of solid tumors (e.g. ovarian, breast, and pancreatic). Taxol acts by hyper-stabilizing microtubules, inhibiting mitosis, and eventually causing cell apoptosis. The change in cytoskeleton dynamics likely influences the cell’s ability to apply force and change shape; both are required for metastatic invasion. We have previously shown that the mechanobiological invasiveness of metastatic cells can be determined through their mechanical interactions with a synthetic polyacrylamide gel; metastatic cells indent gels in attempted invasion, while benign cells do not.

**Objectives**: In the current study, we reveal reduction due to Taxol treatment in the mechanobiological invasiveness of metastatic cancer cells, as measured by their ability to forcefully indent elastic gels.

**Methods**: We evaluate the mechanical interaction of two human, metastatic breast cell lines, MDA-MB-231 and MDA-MB-468, with a physiological-stiffness, synthetic, non-degradable and impenetrable polyacrylamide gel. We evaluate the effects of Taxol at concentrations of 5-25μM. Using an inverted, epifluorescence microscope we obtain images of the cells and the fluorescent particles embedded at the gel surface. When cells indent the gels, the particles at the indentation location (beneath the cells) are pushed to lower focal depths. The indentation depth is then calculated by the difference in focal depths for each individual cell within a group.

**Results**: Treatment with Taxol rapidly (< 2 hours) reduces the number of indenting cells as well as the indentation depth. Specifically, the cells exhibit smaller indentations and lose their ability to synergistically and collectively apply force. In addition, the...
effect of Taxol increases linearly with drug concentration, and depends on the cancer cells’ metastatic potential.

**Discussion:** Our synthetic gel-platform shows drug-induced reduction in the mechanobiological invasiveness capacity of metastatic cells. This approach may be extended to patient-personalized testing of drugs and optimization of drug-treatment protocols.

**Keywords:** Mechanobiology, Cancer invasion and migration, Breast cancer, Cell-microenvironment interactions
Introduction

Chemotherapeutics are typically targeted at reducing the growth and proliferation of the primary tumor cells, however, treatments against metastases, the main cause of mortality, require more complex approaches. Anti-metastatic drugs may, for example, inhibit: vascularization, cell growth in different microenvironments, cell-extracellular matrix (ECM) binding, various receptor kinases, and cancer stem cells [1,2]. Thus, in clinical cases where metastases are likely, a combined, aggressive treatment targeting both tumor growth and metastasis formation is typically used. However, such aggressive treatments reduce the patients’ quality of life and increase their risk for kidney or liver-related toxicity [3]; inappropriate treatment may even induce metastasis formation. Therefore, accurate assessment of the metastatic risk and subsequent identification of chemotherapeutics that may reduce metastasis formation are critically important. A key step in formation of metastases is the invasion of cancer cells through surrounding tissue, including cells and ECM. Most solid tumors exhibit collective migration during metastasis [4], requiring synergistic interactions between the cells [5]. The molecular mechanisms of cancer invasion depend on cancer phenotype and the microenvironment [6], yet typically include mechanobiological interactions [7], i.e. for cell adherence and subsequent force application during migration and invasion.

Accordingly using mechanobiology, we have shown that the invasiveness of cancer cells, indicative of the metastatic risk, can be rapidly (< 2hrs) identified through their mechanical interactions with an impenetrable gel [5,8,9]. Specifically, we have shown that following cell attachment, mechanically capable subpopulations of cancer cells indent an impenetrable gel in attempted invasion, while benign cells do not indent the gels [5,8,10]; the metastatic cells indent the gels by utilizing elements of the cytoskeleton [10] to simultaneously applying lateral and normal forces to the gels [8]. We have determined that the number of indenting cancer cells and their attained depths correlate with their invasive capacity [5,8]. Furthermore, we have shown that the subpopulations of indenting cells correspond to those that are able to cross an 8μm Boyden chamber membrane [9], thus correlating indentations and their depth with the invasiveness of the evaluated cells.

Until recently anti-cancer and anti-metastatic treatments were chosen and adapted to a patient by trial and error [11]. Recently, new methods and techniques for personalized cancer treatment were developed, all of which have limitations, e.g. costs,
test-times, accuracy, and specificity. For example, drugs were introduced in vivo directly into the tumor, their effect was evaluated by resecting the sample, and the subsequent treatment protocol decided from those results [12,13]. As in vivo experiments are complex and dangerous, mathematical models have been used to determine personalized drug doses [14] and genetic testing has been used to identify patient-subsets that may benefit from a given therapeutic[15,16]. To further identify drugs appropriate for specific patient subsets, the response of a wide variety (>400) of human, cancer cell lines to over 130 anticancer agents was determined [17,18], using DNA, RNA and chromosome-level characterization, as well as drug-sensitivity profiles. While the response of cell lines in in vitro culture to drugs do not accurately reflect the in vivo effects, they are beneficial for preliminary drug-screening [19]. An added value in a chemotherapy would be if it were able to reduce the metastatic capacity of cells.

A commonly used treatment to inhibit growth of various solid cancers (ovarian, breast, pancreatic, etc.) is the chemotherapy drug Paclitaxel (Taxol) [20,21]. Paclitaxel has a relatively high (up to 76%) response rate in patients [22], however has also been shown, in some cases, to have a dose dependent pro-metastatic effects [23,24]. Therefore, it is extremely important to determine appropriate doses through their effect on metastatic capacity of cancer cells. The effect of Paclitaxel is through hyper-stabilization of the microtubules (MTs), whereby eliminating the MT dynamics, mitosis is inhibited [25]. In the context of metastasis, MTs also have important roles in cell motility and division, organelle transport, and in cell morphogenesis and organization. We have previously shown that intracellular motion is actively driven by fluctuating microtubules, and internal dynamics is directly affected by Taxol treatment [26]. In addition to the hyper-stabilization of microtubules, The enhanced polymerization and re-localization of F-actin in Taxol-treated cells was also demonstrated [23,26], which has led to reduced adhesive forces applied by cancer cells on gels [27].

In the current work, we show that using the gel-assay we can determine the efficacy of varying concentrations of the chemotherapeutic drug Paclitaxel in reducing the mechanobiological invasiveness of the evaluated metastatic breast cells. We seed high and low metastatic potential cells on the gels and show a Taxol-concentration dependent reduction in the number of indenting cells and the attained depths. Both the single cells and cells in close proximity (i.e. densely seeded) exhibit reduced indentation depths, yet the effect is more significant when cells are closely situated.
Specifically, the synergistic (collective) ability of the densely seeded breast cancer cells to indent the gels more deeply [5] is lost following the short timescale exposure (1-2 hours) to Taxol in different concentrations. In addition, we show a difference in the response of the high and low metastatic potential cells, relating to their relative invasiveness.

Materials and Methods

Cell culture. We have used two commercially-available human, epithelial breast cell lines: high MP (MDA-MB-231, ATCC, Manassas, VA), low MP (MDA-MB-468, ATCC, Manassas, VA) both breast cancer cells collected from lung metastases. The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% vol. fetal bovine serum (FBS), 1% vol. each of L-glutamine, penicillin-streptomycin and sodium pyruvate (all from Biological Industries, Kibbutz Beit Haemek, Israel) [5,8,9,28–30].

Polyacrylamide hydrogel preparation. Polyacrylamide (PAM) gels were prepared with Young’s modulus 2.4 kPa (stiffness of physiological soft tissue [31]) according to an established protocol [8,28]. Acrylamide (34 µl of 40%vol) and BIS acrylamide monomers (3.8 µl of 2%vol.) were combined (both Sigma, St. Louis, MO), within 203µl of distilled water. Gelation was initiated with ammonium persulfate (APS) and catalyzed with the tertiary aliphatic amine N,N,N’,N’-tetramethylethylenediamine (TEMED, both Sigma-Aldrich, St. Louis, MO). Red fluorescent (excitation/emission 580/605 nm) carboxylated polystyrene particles (Invitrogen, Carlsbad, CA), 200 nm in diameter, were embedded at the gel’s surface by performing slow gelation at 2°C during centrifugation. Finally, the surface of the gel was shortly activated with Sulfo-SANPAH (Pierce, Thermo Scientific, Waltham, MA), washed with HEPES, and coated with rat tail collagen type I (Sigma, St Louis, MO) to facilitate cell adhesion [6-8,25–27].

The Young’s modulus, $E$, of the gels was obtained by the following relation: $E = 2G^*(1 + \nu)$ [8,29], where the Poisson’s ratio, $\nu$, is 0.49 for PAM gels [32]. The $G^*$ was measured using a TA Instruments AR-G2 rheometer (New Castle, DE) with a 2-cm stainless steel parallel plate fixture. Using dynamic measurements we determine that the loss ($G''$) modulus is 1-2 of magnitude less than storage modulus ($G'$). Therefore the
complex modulus, $G^* \approx G'$, as expected for an elastic material [33]. The gel recipe yielded a gel with 2.44±0.04 kPa, with high reproducibility between batches [8,27]. Polyacrylamide gels have previously been shown (by comparing rheology and AFM measurements) to be spatially homogeneous and to possess remarkably linear mechanical properties [34].

**Sample preparation.** We have seeded 300,000 cells per gel, resulting in an average of $19 \pm 5$ cells per field-of-view (area of 0.016 mm$^2$). The cells were closely situated or adjacent on the gel (some touching), yet typically remain in a 2-dimensionsal layer on the gel. Samples (including cells and gels) were immersed in 1ml growth media reaching 1 cm above the gel height. The cells were allowed 40-55 minutes to attach the gels and then drug treatment and/or imaging was performed. Conditions of 37ºC, 5% CO$_2$, and high humidity (90%) were maintained throughout the experiment, using an on-stage and on-microscope incubator (Life Imaging Services, Switzerland), to maintain cell viability for prolonged periods of time.

**Drug treatment.** A stock solution of 0.01M Paclitaxel (Taxol) (Cytoskeleton Ltd., Denver, CO) in Dimethyl Sulphoxide (DMSO, Sigma, St Louis, MO) was diluted with cell-growth media to a final concentration of 5, 10 or 25 $\mu$M, and was added to the cells attached to the gels. The cells were incubated with the drug for 1-2 hours before imaging. At least three independent experiments were performed with each Taxol concentration (including control with no Taxol) for each of the two cell lines, resulting in statistically significant numbers of indenting cells (see Table S1).

**Viability and nucleus staining.** Viability of cells was determined by Calcein-AM fluorometric assay (BioVision, Milpitas, CA) [35], where the compound easily penetrates the intact membranes of live cells. Hydrolysis of Calcein-AM by intracellular esterases produces fluorescence in the cell cytoplasm which can be observed at excitation/emission of 485/530 nm starting at 30 min after staining. Cell nuclei were live-stained using Hoechst 33342 (Sigma, St Louis, MO) [36], which fluorescently labels the nuclei (excitation/emission 346/460 nm) after 1-2 hours of incubation.

**Microscopy and imaging.** The imaging for cell indentations was done using an on-stage and on-microscope incubators with an inverted, epifluorescence Olympus IX81 microscope (Olympus Japan), using a 60x/0.7NA differential interference contrast
DIC air-immersion, long working-distance objective lens. Images were captured using a XR Mega-10AWCL camera (Stanford Photonics Inc., Palo Alto, CA) with a final magnification of 107.8 nm/pixel [8,9].

In each sample, at least 28 different randomly chosen fields of view (FOV) were imaged. We collected images of the cells, stains and the particles embedded at the gel surface, and at different depths where particles were in focus (Figure 1). Specifically, at each FOV we obtained a DIC image of the cells on the gel, fluorescence images of the calcein (viability) and Hoechst (nucleus) stains, a fluorescence image of the particles at the gel surface focal plane, and a series of fluorescence images of the particles at 4-6 focal depths below the gel height, where 1-8 indenting cells were in focus at each depth (Figure 1). The indentation depth was then calculated by the difference in focal depths between the fluorescence image at the gel surface (undisturbed gel) and at the lowest focal plane where particles are in focus, i.e. at the bottom of the specific indenting cell [5]. Images were analyzed using a custom-designed module in MATLAB 2012b (The MathWorks, Natick, MA) to determine the number of viable and indenting cells, as well as the indentation depth of each cell. We determined the number of indenting cells out of the total viable cells.

**Statistical analysis.** The cells with high or low metastatic potential at the same condition were compared, as were the cells of the same MP but under different conditions. The general, linear mixed model, which is a multivariate regression method that helps to generalize the analysis of variance (ANOVA) was used [37]. In all cases, statistical significance was determined when the p-value is less than 0.05.

**Results**

We have seeded high and low MP breast cancer cells on 2.4kPa PAM gels, allowed attachment for 40-55 minutes, and evaluated changes in their mechanical interactions following 1-2 hours treatment with Taxol. Cells were seeded in amounts that resulted in many groups of adjacent yet non-overlapping cells in each field of view [5]; while cells were in close proximity and some were in contact they remained in a 2-dimensional layer on the gel surface. A subpopulation of the metastatic cells indented the gels before drug treatment. The percentage of untreated (0µM Taxol) indenting, closely situated high and low MP cells was 61±10% and 38.8±8.9%, respectively, matching our previously published work [5]; untreated single, well-spaced cells indent
in smaller amounts, respectively, 49±8% and 33±5% [9]. Cell morphology was unaffected by the Taxol treatment and the cells remained rounded (Figure S1), in contrast to the same cell lines on a rigid, glass surface, which become rounded with protrusions on their surface [26]. We have evaluated the change in cell viability, the number of indenting cells and their depths, following treatment with 5, 10 and 25µM Taxol as compared to untreated control (Figure 2 and Table S1).

Viability of the high MP (MDA-MB-231) and the low MP (MDA-MB-468) breast cancer cells was affected by higher concentrations of the Taxol. Both cell types were not significantly affected by, respectively, ≤5µM and ≤10µM Taxol treatment (Figure 2A and Table S2). At the highest tested concentration of 25µM Taxol the viability decreased significantly for both cell lines, yet the low MP cells were more affected; these cells were shown to undergo programmed cell death by Taxol treatment [38]. This may relate to weaker cell adhesion after 2 hours on the soft, elastic gels, as we have previously shown these cells’ viability is uninfluenced by 25µM Taxol when they have been adhered on glass for 24h [26]. The subpopulation of adhered and viable cells that indent the gels are the focus of the current study.

We observe that the percentage of indenting cells of both types reduces linearly with increasing Taxol concentration (Figure 2B); the difference is statistically significant between most concentrations (Table S3). The reduction is steeper in the high MP cells, yet more high MP cells indent the gels than the low MP cells, at all Taxol concentrations (Table S3). Interestingly, we note that following 25µM Taxol treatment, the percentage of indenting high MP cells reduces to a similar amount as the control, untreated low MP cells.

The reduced amounts of indenting cells also decrease their attained indentation depths following Taxol treatment (Figure 3). As we have previously shown [5], the baseline indentation depth of the untreated, breast cancer cells in close proximity is a two-peak distribution, varying between 2-20 µm with the minima at 10µm (Figure S2). The minima depth of 10 µm is, interestingly, also the maximal indentation depth exhibited by single, well-spaced cells [5,8,9]. We observe that Taxol treatment significantly reduced and even eliminated the number of cells that indent the gels >10 µm (Figure 4); the depth distributions become similar to those of single cells as observed e.g. in well-spaced, single high MP cells under 25µM of Taxol (Figure S3). The high MP cells are more rapidly affected, reaching a plateau response at >10 µm
Taxol, yet the means of the depths <10 µm are unchanged. In contrast, in the low MP cells, where less cells indented to deep depths to begin with, the effect of the treatment was milder.

**Discussion**

The morphologies of the cells during gel indentation correlate with those previously observed during cancer cell invasion *in vitro* and *in vivo*; here and also previously we have observed rounded cell morphologies on the soft gels, which appear mushroom-like in 3D [8,10]. Various stages and types of cancer cell invasion initialize with the cells changing shape and applying mechanical forces to their surroundings. Specifically, during the initial steps of *in vivo* cancer cell invasion into the stroma, penetration through the ECM and endothelial transmigration (intravasation/extravasation) invading cells must change morphology according to the locally encountered microenvironment; cells squeeze through small pores by narrowing their leading edge and/or by applying force. Specifically, leading cells (i.e. tip cells) typically exhibit pseudopods and similar protrusions, observed during invasion both *in vitro* and *in vivo* [39–42]. Hence, the morphology of invading cells that develops during their mechanical interactions with the gel substrate (quantified through the indentation depth) can provide a measure for the cells’ invasive capacity.

We have observed a reduction in percentage of indenting cells and in the attained indentation depths – their mechanobiological invasiveness, which correlates with the cells migratory and invasive capabilities [9]. Similar effects of comparable concentration Taxol treatments on cancer cells have previously been observed, yet on longer time scales. The number of migrating low MP breast cancer cells (MDA-MB-468) treated with 3.12µM Taxol was dramatically reduced, as measured by a gap-closure assay [43]. The invasive capacity of the cells through narrow barriers was also reduced under 16µM Taxol [44], likely since the cells were unable to dynamically modify their cytoskeletons [10,26]. Correspondingly, adhesion was also affected, as MDA-MB-231 cells trypsinized for 35 min resisted detachment, following 24h Taxol treatment [15]. In contrast, short time-scale Taxol treatment (0.5-1.0µM for 1h) of prostate tumor cells (PC3) was enough to completely inhibit attachment and block migration through a Boyden chamber [45]. Thus, Taxol effects *in vitro* depends on
Taxol concentration and treatment time, as well as on (cancer) cell type and the metastatic potential. We observe higher baseline mechanobiological invasiveness in the high MP breast cancer cells, yet also a larger reduction following Taxol treatment; the high MP cells still always remain more invasive than the low MP cells. We have previously shown that the microtubule network is sparser in the high MP cell lines when on glass [26,46], which is likely why their response to the microtubule-targeting drug is stronger. We have observed that during indentation, both cell types exhibit cell-wide dispersion of the microtubules at times concentrated at the leading edge, where the actin is localized [10]. The microtubule structure and dynamics in both cell types is affected by Taxol treatment [26], where the dynamics are reduced. This directly affects the cells’ ability to dynamic modify their internal structure and actively apply force to the gels.

We observe that under Taxol concentrations of 5-10µM applied up to 5 hours the in vitro mechanobiological invasiveness is reduced while the cell viability is not significantly affected. Mechanobiological changes following microtubule disruptions have previously been shown to be on short timescales, up to 1 hour [47,48], enabling rapid, survival-driven responses. In contrast, growth inhibition is typically on longer timescales than evaluated here; growth inhibition is assessed on timescales comparable to the doubling time of cells. In vivo, Taxol exposure is also on longer timescales and concentrations are typically lower. The IC50, concentration at which 50% of cell growth inhibited of the two evaluated breast-cancer cell lines has been determined as 1-100 nM in different studies [49–51], being highly dependent on the exposure time, typically 24-48 hours. Interestingly, high concentrations of Taxol (on the order of 10µM) did not inhibit cell growth more than lower concentrations [49] and in fact increased cell survival [51]. Similarly, low Taxol concentrations applied for long times (20nM for 24 hours) have also shown a reduction in adherence forces of the high MP cells (MDA-MB-231). Specifically, the lateral, 2-dimensional traction forces (in plane with the gel surface) reduced to 1/3 of the untreated control [27]; traction forces of cells adhered for 10 hours [27] are larger than applied by the same cells during adherence (1-6 hours) [29].

The mechanobiological response combined with changes in viability also control the effects in vivo and the overall effectiveness of the drug; cell damage and death following chemotherapy directly affect the function of neighboring cells. Products of injured and dead cells in the tumor environment induce activation of the toll-like
receptor-4 (TLR4) in viable cancer cells, which has been correlated to mechanisms for Paclitaxel chemoresistance [24]. Concurrently, pro-tumorigenic effects have been induced by chemotherapy damaged tumor cells [52] by contributory host effects [53]. In cells that undergo apoptosis due to chemotherapy, caspase-3 is activated causing upregulation of prostaglandin synthesis and release of growth-promoting factors, which promote tumor-site repopulation by the surviving tumor cells [52,54]. Thus, for in vivo applications, the optimal drug concentration is one that will reduce the cell invasiveness while not reducing their viability.

Conclusion

In conclusion, we have shown that the mechanobiological invasiveness of the evaluated high and low MP breast cancer cells reduces following Taxol treatment, while the viability is nearly unaffected. That is, the ability of the cells to indent the cells is reduced and synergistic interactions between the adjacent cells is lost, albeit the viable high MP cells are still able to indent more than the low MP cells at all evaluated Taxol concentrations; evaluation of more cell lines would be required to determine the generality of this phenomenon. Hence, using our previous correlation of the indentation depth with invasiveness of the cells [9], we conclude that the Taxol reduces the invasiveness of the cell lines evaluated in the current study. Thus, the gel-deformations (e.g. indentation) that are induced by cell-applied forces can reveal the reduction in cell invasiveness due to chemotherapy. Using our mechanobiology approach, we have identified specific drug concentrations where cell viability is maintained while the mechanobiological invasiveness of the cells on physiological stiffness gels is reduced. Current drug-screening assays (typically with the cell viability as the only parameter to determine the drug-response) are mostly performed on rigid tissue culture plates. However, physiological stiffness is likely more appropriate, affecting design of predictive biomaterial-based in vitro assays for drug screening [55]. Using our tunable stiffness gels [8,29] and evaluating cell viability concurrently with mechanobiological invasiveness may provide an approach to determine the optimal drug concentrations and dosages in a cell line specific or patient-personalized approach.

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Figures

Figure 1: Representative images of high MP cells (MDA-MB-231) indenting a 2.4 kPa polyacrylamide gel, no added Taxol. (A) DIC image of cells on gel; (B) live staining of the cytoplasm (green) and nucleus (blue); (C) fluorescent, 200-nm beads at the gel surface, out of focus regions indicate that cells are indenting the gel at that location, displacing the beads to a lower focal plane; (D) at a representative depth of 6.4µm below gel surface, the beads underneath arrow marked cell locations are in focus, other cells in the field-of-view indent to different depths. Scale bar is 20µm.
**Figure 2**: Effect of 1-2 h Taxol in varying concentrations on viability and indentation capacity of cells seeded on 2.4 kPa polyacrylamide gels. (A) Cell viability is not significantly affected at the low concentrations during the evaluated timescales, but reduces at ≥10μM for high (green, left) and at 25μM for low (red) MP cells (see Table S2). (B) Percentage of indenting high (●) and low (▲) MP cells reduces significantly with Taxol concentration and is significantly higher in the high MP cells relative to the low MP cells at all concentrations (see Table S3). Solid lines are linear regressions (R² > 0.98) with slope of (-1.06) for high MP cells and (-0.72) for low MP cells. Error bars are standard deviations.
Figure 3: Distribution of indentation depths attained by high MP (A) and low MP (B) indenting cells on 2.4 kPa polyacrylamide gels at each Taxol concentration (see also Figure S2 and Table S1). Control, untreated cells exhibit a second indentation peak at depths >10 μm, including 64% and 11% of the high and low MP cells, respectively; the vertical dashed line indicates the 10 μm minima. The percentage of deeply indenting cells reduces with Taxol concentration while the average depth of the peak <10 μM remains statistically unchanged (Table S1).
Figure 4: The percentage (from total indenting cells) of high (●) and low (▲) MP cells that indent deeper than 10µm, within the second peak, likely resulting from synergistic cell interactions.