INTRODUCTION

The spreading of primary tumors to secondary sites (tumor metastasis) is a complex process that involves multiple, sequential steps. Vascular adhesion and extravasation of circulating tumor cells (CTCs) is one, critical step. Curcumin, a natural compound extracted from Curcuma longa, is known to have anti-tumoral, anti-proliferative, anti-inflammatory properties and affect the expression of cell adhesion molecules, mostly by targeting the NFκB transcription factor. Here, upon treatment with curcumin, the vascular behavior of three different estrogen receptor negative (ER−) breast adenocarcinoma cell lines (SK-BR-3, MDA-MB-231, MDA-MB-468) is analyzed using a microfluidic system. First, the dose response to curcumin is characterized at 24, 48, and 72 h using a XTT assay. For all three cell lines, an IC50 larger than 20 μM is observed at 72 h; whereas no significant reduction in cell viability is detected for curcumin concentrations up to 10 μM. Upon 24 h treatment at 10 μM of curcumin, SK-BR3 and MDA-MB-231 cells show a decrease in adhesion propensity of 40% (p = 0.02) and 47% (p = 0.001), respectively. No significant change is documented for the less metastatic MDA-MB-468 cells. All three treated cell lines show a 20% increase in rolling velocity from 48.3 to 58.7 μm/s in SK-BR-3, from 64.1 to 73.77 μm/s in MDA-MB-231, and from 57.5 to 74.4 μm/s in MDA-MB-468. Collectively, these results suggest that mild curcumin treatments could limit the metastatic potential of these adenocarcinoma cell lines, possibly by altering the expression of adhesion molecules, and the organization and stiffness of the cell cytoskeleton. Future studies will elucidate the biophysical mechanisms regulating this curcumin-induced behavior and further explore the clinical relevance of these findings.

Keywords: circulating tumor cells, vascular adhesion, parallel plate flow chamber, curcumin treatment, metastasis
endothelial receptors (ICAM-1, VCAM-1), and different families of integrin molecules (αvβ3, integrins; Wirtz et al., 2011; Geng et al., 2012). In organs with high occurrence of metastasis, such as the liver, this picture is even more complicated in that the characteristic discontinuous and fenestrated endothelium leaves the underlying extracellular matrix directly accessible to CTCs. Moreover, vascular adhesion is also supported by the reorganization and deformation under flow of the cell cytoskeleton. Although tumor cells do not exhibit a leukocyte-like cortical cytoskeleton which is capable of extensive and rapid deformation, it is possible that CTCs exposed to shear stresses in the circulation could undergo transformations facilitating attachment to the vessel walls (Davies et al., 2005). Indeed, firm arrest is a necessary condition for the subsequent extravasation and colonization of the surrounding tissue.

The development of therapeutic agents against metastatic disease is still in its infancy, due to a lack in understanding the leading pathways and, most importantly, their alterations in secondary tumor cells. Also, most of the efforts have been traditionally oriented to eradicate tumor cells already proliferating at the secondary sites, neglecting the opportunity of blocking or modulating one or multiple steps in the metastatic cascade (Chambers et al., 2000). Novel micro- and nanotechnologies detecting, capturing, and characterizing CTCs are providing new and more accurate information on the biomechanical properties of malignant cells in the circulation. Microfluidic systems used as diagnostic tools are demonstrating the significant correlation between higher CTC counts in blood and lower patient survival (Cristofanilli et al., 2004; King et al., 2009; Han et al., 2010; Hughes and King, 2012). It is then reasonable to speculate that novel therapies, possibly nanoparticle-based, could open new avenues for an effective treatment of metastatic diseases by eradicating, or dramatically lowering, the number of CTCs.

In this work, the effect of curcumin on the rolling and adhesion mechanics under flow of three estrogen receptor (ER−) breast adenocarcinoma cell lines, namely SK-BR-3, MDA-MB-231, and MDA-MB-468, is analyzed. Curcumin is a natural multi-target compound with anti-tumoral and anti-inflammatory properties (Kumar et al., 1998; Ray et al., 2003; Kunnumakkara et al., 2008; Binon et al., 2009; Yodkeeree et al., 2010). Their rolling velocity and adhesion propensity are measured experimentally upon treatment with curcumin, using a parallel plate flow chamber system.

**MATERIALS AND METHODS**

**MATERIALS AND CHEMICALS**

SK-BR-3, MDA-MB-231, MDA-MB-468 breast adenocarcinoma cells were obtained from the American Type Cell Culture Collection (ATCC) and cultured in the recommended medium. SK-BR-3 were grown at 37°C, under a humidified 5% CO2 and 95% air at one atmosphere, MDA-MB-231 and MDA-MB-468 were grown at 37°C in a free gas exchange with atmospheric air. Collagen type I from calf skin was obtained from Sigma Aldrich (St. Louis, MO, USA). Curcumin (diferuloylmethane) and dimethyl sulfoxide (DMSO) were obtained from Fisher Scientific. XTT kit was obtained from Trevigen (Gaithersburg, Maryland).

**CYTOTOXIC EFFECT OF CURCUMIN AND XTT ASSAYS**

Curcumin was first dissolved in DMSO as a 10 mM stock solution and subsequently diluted in cell culture medium. Medium containing the same amount of DMSO was used as control at concentrations not exceeding the 0.1% v/v of the culture medium. Cells were incubated with curcumin at 1, 10, 20, and 40 μM, at three time points namely 24, 48, and 72 h. Cell proliferation was measured with conventional XTT reduction assays. Briefly, SK-BR-3, MDA-MB-231, and MDA-MB-468 cells were inoculated at a density of 5 × 10^4 cells in 96-well plates for 24 h in 200 μl of recommended medium. The culture supernatant was then removed and medium containing the above mentioned curcumin concentrations was added to cells, subsequently incubated for 24, 48, and 72 h. After that, XTT-dye was mixed with phenol-free medium and added to the samples. The plate was incubated for at least 1 h before reading. The absorbance of XTT-formazan dye was then measured using a microplate reader at 490 nm. Twenty-five repetitions were performed for each time point and concentration.
Before flow adhesion experiments, cells were first incubated with medium containing no FBS for 7 h and then treated with or without curcumin at 10 μM for 24 h. Subsequently, cells were detached from culture dishes by mild trypsinization (0.25% trypsin/EDTA) for 2 min at 37°C and incubated at 37°C for 1 h to allow the regeneration of surface glycoproteins. After that, cells were washed in PBS and resuspended at 106 cells/ml in serum-free medium containing 0.1% bovine serum albumin, following standard protocols (McCarty et al., 2000).

The rolling and adhesion behavior of the tumor cells, was studied using a parallel plate flow chamber system (Chen et al., 1997; Brown and Larson, 2001; GlycoTech Corporation; Figure 2). The system comprises a commercially available flow chamber, a syringe pump (Harvard Apparatus, MA), an inverted epifluorescent microscope (Nikon Ti-Eclipse) and a desktop computer for data storage and analysis. The main constituents of the flow chamber are a deck, a rubber gasket and a glass slide. The rubber gasket defines the geometry of the flow region (length \( l = 20 \text{ mm} \); width \( b = 10 \text{ mm} \); height \( h = 0.254 \text{ mm} \)). The coverslips (slide), closing the bottom of the chamber, were covered with a uniform collagen layer. In particular, autoclaved 35 mm coverslips were covered by a collagen solution obtained diluting collagen type I from calf skin (Sigma Aldrich) in PBS to reach a concentration of about 50 mg/cm².

After assembling all the components, the system was placed on the stage of an epifluorescent microscope (Nikon Ti-Eclipse). The Andor’s Luca EM S camera utilizes a 658 × 496 “interline frame transfer” EMCCD sensor to acquire the region of interest (ROI) and allows for real time monitoring. For each experiment 10⁶/ml cells were perfused in 1 ml of serum-free medium, at a wall shear rate of \( S = 10 \text{ s}^{-1} \) (mimicking the circulation environment of microvascular tumor vessels). Eight experiments were performed for the SK-BR-3 and MDA-MB-468-treated and untreated cells, and 10 experiments for the MDA-MB-231. All the experiments lasted 12 min each.

Through offline data analysis on the movies derived from each experiment, the number of firmly adhering cells on the substrate and their mean rolling velocity were quantified. Firmly adhering cells were those cells staying within the region of interest (10× objective, ROI \( = 658 \times 496 \text{ pixel} \)) till the end of the experiment. This number was normalized by the total number of injected cells and the area of the ROI \( (\sim 0.33 \times 10^{-6} \text{ m}^2) \) deriving the adhesion propensity. The rolling velocity was calculated as the displacement of the centroid of the cells divided by the time interval of their observation (on average about 10 s). The rolling velocity was calculated for 12 cells in three different SK-BR-3 experiments \( (n = 36) \), and six cells in eight different MDA-MB-231 and MDA-MB-468 experiments \( (n = 48) \), for both the treated and the control group.

**RESULTS AND DISCUSSION**

The cytotoxic effect of curcumin on SK-BR-3, MDA-MB-231, and MDA-MB-468 cells was analyzed using a XTT proliferation assay. Cells were incubated with curcumin at different doses, namely 1, 10, 20, and 40 μM, and for three time points, namely 24, 48,
and 72 h. The cell viability was measured following the proto-
col procedures described in Section "Materials and Methods." and is reported in the bar charts of Figure 3, for 25 repetitions in each cell line. As expected the percentage of viable cells reduces as the concen-
tration and the duration of the treatment increase. Interestingly,
for concentrations lower and equal to 10 μM, curcumin has no significant effect on the cell viability. Note that for the con-
trol experiments, cell culture medium was added with the same
amount of DMSO used in the actual experiments for dissolving
curcumin. This volume was about 0.1% of the total medium vol-
ume and no toxicity was observed on the cells. For larger
concentrations, 20 and 40 μM, the curcumin treatment limits cell
proliferation in a dose and time dependent manner. Cell viabilities
lower than 50% can only be observed at the highest concentra-
tion and longer time points (48 and 72 h). The IC_{50} is reached at ~20 μM for the SK-BR-3 and MDA-MB-468 at 72 h, and at ~40 μM for the MDA-MB-231 at 72 h. This is in agreement
with most literature on curcumin (Aoki et al., 2007). From this
assay, the exposure to 10 μM of curcumin for 24 h was consid-
ered a mild treatment inducing no significant, direct effects on cell
viability.

The effect of curcumin on the rolling and adhesion behavior of
the three breast adenocarcinoma cells lines SK-BR-3, MDA-MB-
231, and MDA-MB-468 was analyzed using a parallel plate flow
chamber, traditionally employed for the analysis of leukocyte adhe-
sion and rolling (Lawrence et al., 1987). The cells, mildly pretreated
with curcumin (24 h at 10 μM) as detailed in Section "Materials
and Methods," were infused in the apparatus depicted in Figure 2
and their rolling velocity and firm adhesion was quantified via
post processing of the images taken with an epifluorescent micro-
scope. The bar charts of Figure 4 present the results of the flow
chamber assays. Figure 4A shows the adhesion propensity of the
curcumin-treated cells (Curc-treated) and untreated cells (Ctr), as
the ratio between the absolute number of adhering cells (n_{ad}), the
number of injected cells (n_{ini} = 10^6), and the area of the
region of interest (A = 0.33 x 10^{-5} m^2). SK-BR-3, MDA-MB-231,
and MDA-MB-468-treated cells present an adhesion propensity of
90.7 ± 28.2, 58.56 ± 19.70, and 132.97 ± 31.34#/m2, respectively.
This means that for a vessel of 50 μm in diameter with a 300 μm length
(vascular area ~1 mm²), about 300 SK-BR-3, 180 MDA-
MB-231, and 400 MDA-MB-468 cells would firmly adhere under
the same biophysical conditions and assuming that 10^6 CTCs enter
the specific vessel. On the other hand, the untreated cells exhibit a
larger adhesion propensity of 151.1 ± 60.84, 109.69 ± 38.19, and
155.42 ± 20.74#/m² for the SK-BR-3, MDA-MB-231, and MDA-
MB-468, respectively. The difference between the two populations
(Curc-treated and Ctr) is significant for the SK-BR-3 and even more
for the highly metastatic MDA-MB-231 cells. More specif-
ically, the curcumin-treated SK-BR-3 cells show a 48% decrease
(p = 0.02) in adhesion and the curcumin-treated MDA-MD-
231 a 47% decrease (p = 0.001) as compared to the control
group. Conversely, the difference in adhesion propensity between
curcumin-treated MDA-MB-468 cells and the control group is not
statistically significant (p = 0.099).

Rolling velocity is reported in Figure 4B. This physical quan-
tity was estimated as the ratio between the displacement of the cell
centroid over the corresponding observation time. Despite the
large standard deviation, this analysis shows that there is a signifi-
cant difference between the two groups (Curc-treated and Ctr) in
all three cell lines: SK-BR-3 and MDA-MB-231-treated tumor cells
roll ~1.2 times faster than the control cells (p = 0.01, p = 0.009,
respectively), MDA-MB-468-treated tumor cells rolls ~1.3 times
faster than the control cells (p = 0.0005). In particular, the rolling
velocities are 58.66 ± 20.29 and 48.33 ± 11.3 μm/s for SK-BR-
3-treated and untreated cells, 73.77 ± 21.21 and 64.1 ± 12.89
for MDA-MB-231-treated and untreated cells, 74.38 ± 24.88 and
57.49 ± 11.96 for MDA-MB-468-treated and untreated cells,
respectively. Indeed, the higher rolling velocity correlates well with
the lower adhesion propensity observed above.

These preliminary results collectively would suggest that mild
treatments with curcumin could impair cell adhesion and increase
cell rolling under flow over normal, untreated cells. Indeed, this
could reduce the metastatic potential of CTCs. Understanding
the mechanisms regulating the observed alternation in the behav-
or of SK-BR-3, MDA-MB-231, and MDA-MB-468 cells is out of
the scope of this preliminary study. However, it has already been
reported that curcumin treatments alter the organization of micro-
filaments and increase the overall quantity of F-actin. This would
affect cell motility and deformability, which are crucial elements
in supporting tumor cell circulation and survival in the blood
stream. Moreover, recently it has been shown that CTCs reattach in
distant tissues by a mechanism that is tubulin-dependent and sup-
pressed by polymerized actin (Holy, 2004; Matrone et al., 2010).
In addition, curcumin is known to decrease the expression and

![Figure 4A](https://example.com/figure4a.png)

![Figure 4B](https://example.com/figure4b.png)

![Figure 4C](https://example.com/figure4c.png)

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modulate the activity of membrane adhesion molecules by acting on the transcription factor NF-κB. For instance, the curcumin inhibition of NF-κB completely blocked TNF-α induced expression of adhesion molecules (ICAM-1, VCAM-1, and E-selectin) on HUVECs and human intestinal microvascular endothelial cells attenuating leukocytes adhesion (Kumar et al., 1998; Ray et al., 2003; Binion et al., 2009). Therefore, in the present case, it is reasonable to speculate that curcumin effects could depend, at least in part, on a reduced expression or more likely, on the modulation of integrins receptor activity (mostly α1β1 and α2β1) thus limiting the adhesion propensity under flow (Park et al., 2006; Ivascu and Kubbies, 2007). Here, it is important to note that integrins, expressed on cellular membrane, can specifically bind to the collagen type I, deposited on the flow chamber glass slide. Also, in organs with high metastatic occurrence, such as the liver, the discontinuous, fenestrated endothelium allows the CTCs to directly interact and bind to ECM components. For this reason, collagen type I, has been also used in flow chamber experiments to assess the adhesive behavior of cells and CTCs (Haier et al., 1999; Wendel et al., 2012).

Finally, curcumin, a natural compound extracted from Curcuma longa, has been demonstrated to have a wide spectrum of biological and pharmacological activities. In particular, it exhibits antiviral, antibacterial, antioxidant, anti-inflammatory, anti-proliferative, and anti-angiogenic properties (Aggarwal et al., 2003; Holt et al., 2005). Animal and human studies have suggested its potential use in the treatment of inflammation and cancer, mostly because of its potent effect on the NF-κB pathway (Kessamsetti et al., 1999; Aggarwal, 2004). However, the major drawback in its clinical use is the very low bioavailability and biodistribution, mostly due to its poor absorption from the gut, rapid metabolism and elimination (Shoba et al., 1998; Anand et al., 2007). The formulation of curcumin into nanoparticles could avoid the drawbacks listed above and enhance its curative properties.

**CONCLUSION**

The ER negative breast metastatic cells, SK-BR-3, MDA-MB-231, and MDA-MB-468, cells were treated with curcumin, at three different time points. For sufficiently large curcumin doses (≥ 20 μM), significant cell death is induced at 72 h. Conversely, a mild treatment with curcumin (≤ 10 μM at 24 h), did not show any significant change in cell viability but did affect the vascular behavior of the cells. This was demonstrated by assessing the cell adhesion and rolling velocity in a parallel plate flow chamber system. The SK-BR-3 cells showed a 40% decrease (p = 0.02) in cell adhesion propensity and 20% increase (p = 0.001) in rolling velocity. The MDA-MB-231-treated cells showed almost a 50% decrease (p = 0.001) in cell adhesion propensity and about 15% increase (p = 0.009) in rolling velocity. The MDA-MB-468-treated cells did not show any statistically significant decrease in adhesion propensity, but did roll 1.3 times faster than the control group (p = 0.00005).

Collectively, these results suggest that mild curcumin treatments of CTCs could lower or even prevent the occurrence of metastasis, by reducing CTCs adhesion at secondary vascular sites. Future works will have to elucidate the mechanisms reg-ulating the observed alteration in tumor cell behavior and the specific pathways involved in each cell line studied, by characterizing the expression and activity of cell membrane receptors, the organization of the cell cytoskeleton and its deformability. However, the proper delivery of sufficient doses of curcumin
to CTCs could provide a new strategy to prevent the metastatic spread.

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