Curcumin Effect on Invasive Behaviour of Triple-negative Breast Cancer Cells

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Curcumin inhibits cell proliferation, metastasis and angiogenesis and also initiates apoptosis; beside these well documented effects, curcumin influences adhesive proteins behaviour in tumor cells. The aim of the present study was to evaluate the dose response effect of curcumin in continuous/discontinuous treatment on the invasive ability of mesenchymal phenotype triple negative breast cancer cells (MDA-MB-231) versus breast cancer cells expressing E-cadherin (MCF-7). Wound closure for MDA-MB-231 cells was noticed to be better for discontinuous curcumin treatment while for the continuous treatment (48+96h) the wound closure was impaired on more extended areas; this observation supports the curcumin involvement in reversing EMT, by inhibiting the invasive phenotype of these cancer cells. All observations were consistent either for discontinuous (48h) and continuous treatment (48+96h). Differences between wound healing rates in the two depicted conditions are enhanced for curcumin concentrations ranged from 7-10μM. Cell viability determined by Cell-Titer-Glo assay showed values between 80.1-97.2% for all curcumin dosage used, including the continuous treatment. The present study shows the low-dose curcumin effect on the migratory ability of triple negative breast cancer cells in culture. The results emphasize the differences between continuous and intermittent treatment of breast cancer cell lines with curcumin concentrations ranged from 1-10 μM. The best results, with consistent correspondence between migration ability inhibition without reducing cell viability below 95% were obtained by a continuous treatment with 5 μM curcumin

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Curcumin, a turmeric ingredient and yellow spice derived from Curcuma longa is broadly used for antioxidant, anti-inflammatory and antineoplasic known effects (prevention and treatment). Curcumin, chemically known as diferuloylmethane ($C_{21}H_{20}O_6$), acts on multiple molecular targets specific for tumor cells [1]. Curcumin action on adhesive proteins is well documented too [2, 3]. Cell membrane glycoproteins that are forming CAMs (cell adhesion molecules) participate to cell-cell or cell-extracellular matrix interactions. It was noticed that NF-κB (nuclear factor kappa B) is partially regulating CAM expression [4]. Curcumin inhibits α6β4 integrin that controls cell mobility in breast cancer but also PI3K/AKT-dependent tumor cell invasion [5].

Curcumin inhibits cell proliferation, metastasis and angiogenesis and also initiates apoptosis [6, 7]. It may induce ROS (reactive oxygen species) generation and DNA damage in tumor cells. Another pro-apoptotic pathway includes anti-apoptotic protein inhibition by curcumin (Bcl2 and BclXL) in pulmonary and pancreatic tumor cell lines. Curcumin demonstrated itself useful against multiresistent tumor cells on classic antitumor compounds [8-11].

Other compounds like betulin possess biological activities as anti-inflammatory, antiviral, and anticancer actions and free fatty acids often have the potential to reduce the blood concentration of low-density lipoproteins, therefore it was found that betulin-fatty acid mixtures with a good size, stability, and no skin irritation potential show cytotoxic potential [12]. Also, series of tetra-substituted zinc(II)phthalocyanines with four 4(3H) quinazolinone ring system units (qz)(4)ZnPcs 4a-c, have been studied for their in-vitro antitumor activity on breast adenocarcinoma (MCF-7), the structure-activity relationship showed that electronic factors in the 4(3H)-quinazolinone moiety that attached to the ZnPc skeleton had a magnificent effect on the antitumor activity of the newly synthesized (qz)(4)ZnPcs 4a-c [13].

The aim of the present study was to evaluate the dose response effect of curcumin on the invasive ability of mesenchymal phenotype triple negative breast cancer cells (MDA-MB-231). These cells were selected due to increased motility potential and ability to reestablish confluence following a scratch-induced defect (wound).

Experimental part

The present experiment assayed the effect of a linear increasing concentration of curcumin on the invasive ability of two breast carcinoma cell lines. Cells have been selected according to their ability to express E-cadherin, main protein involved in intercellular adhesion. MDA-MB-231 cell line is constitutively lacking E-cadherin expression while MCF-7 cell line is natively expressing tis adhesion protein.

Reagents

All reagents were purchased from Sigma-Aldrich. Curcumin (Cat: C1386) as powder was dissolved in pure ethanol to a stock concentration of 2.7mM. Dulbecco's phosphate buffer saline (DPBS), no calcium or magnesium salts (Cat: D8537), and RPMI1640 (Cat: 7509), with sodium bicarbonate, 1% Glutamax, no phenol red, with 10% FCS (fetal calf serum) were used according to manufacturer recommendations and recipes.

Monolayer cultured cells

Cell lines and culture methods

MDA-MB-231 cell line was grown and proliferated in 75cm^2 sterile, cell culture ready flasks, using DMEM (Sigma

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Cat: D6046) low-glucose culture media supplemented by 10%FCS, 1% Glutamax, 1%NEAA (non-essential aminoacids) and 1% antibiotic/antimycotic. MCF-7 cells were grown and proliferated in RPMI1640 (Cat: 7509) with 10% FCS, 1% Glutamax and 1% antibiotic/antimycotic.

Cell detachment was performed by TrypLE trypsin (2mL for 3 min in a 75cm^2 cell culture flask, at 37°C), while trypsin inactivation was performed by 10 mL complete culture media. Cells were gently mixed to detach them from clumps and counted by an automated cell counter (Countess Invitrogen) prior to dispensing into the wells of a 96-well plate. Cell density in the 96-well plate was of 1×10^4 cells/well. Plates with cells and flasks were incubated at 37°C, 5% CO_2 and 99.6% Relative Humidity.

Treatment

Cell growth and proliferation was performed separately for the two cell lines prior to curcumin treatment. Passages were performed at 80% subconfluence. At passage 7, cells from each of the two cell lines were individually treated in separate flasks with curcumin concentrations from 1-10μM. For discontinuous treatment, cells were treated by curcumin concentrations ranged from 1-10μM for 48h. For the continuous treatment, the exposure to curcumin was extended for 96 more hours, the curcumin dissolved in media being replaced daily for each flask. After curcumin incubation, cells in each flask were detached by TrypLE, washed in DPBS, and dispensed in a 96-well plate. In each well we have dispensed 5×10^5 cells in 200uL complete RPMI. At 24h, the wound assay was performed on each monolayer in the wells, using a 200μL tip. Wound healing in each well was monitored and results recorded. Each of the two experimental conditions was replicated 3 times. Experiment was interrupted when MDA-MB-231 cells in the untreated wells reached visually full wound healing.

Assays

Viability assays

Cell viability at passages and prior to cell dispensing in 96 well plates was performed by TrypanBlue assay, described by Tennant Jr et al in 1964 [14].

CellTiter-Glo Luminescent Cell Viability Assay (Promega) was used to assess ATP levels in the cells from treated wells. Manufacturer recommendations and concentrations have been used. Results expressed in RLU (relative luminescence units) were converted to percent viability.

Wound assay

Wound assay or the scratch test was performed according to Liang et al. [15] recommendations. This cheap and feasible assay supposes scratching the subconfluent cell monolayer in a well by a 200 μL tip in order to generate a defect that can be microscopically measured in bright-field microscopy.

Microscopy and image analysis

Images were captured by an Olympus BX80 microscope in bright-field, using the 10x objective. Images were saved and processed by Fiji_ImageJ open-source package [16] (including specific plugins as ABSnake). Results were processed and graphically represented by curve-fitting in GraphPad-Prism 6.0 and Microsoft Excel.

Results and discussions

Control samples were separately represented (fig. 1) while numerical values were represented in the corresponding treated-samples graphics. Full closure of the scratch wound occurred in 48 h for MDA-MB-231 cells in the control samples. These cells have an increased proliferation rate and motility due to the lack of E-cadherin expression. Even though day0 wound areas are slightly different due to the scratch protocol, the variations are not going over 3.6%. For the MDA-MB-231 cells treated by curcumin concentrations from 1-10 μM for 48h, the wound closes less as curcumin levels were higher (fig. 2). The EC50 (the concentration at which curcumin gives a half-maximal response) for MDA-MB-231 cells on day 2 is 8.4 μM (fig. 3). For MCF-7 cells, the EC50 is only 4.9 μM but those cells are natively expressing E-cadherin and the wound closure effect ranged from 7.3×10^5 - 1.1×10^6μm^2 (fig. 4); the wound area on MDA-MB-231 cells ranged from 1.0×10^6-1.0×10^5μm^2. Wound closure for

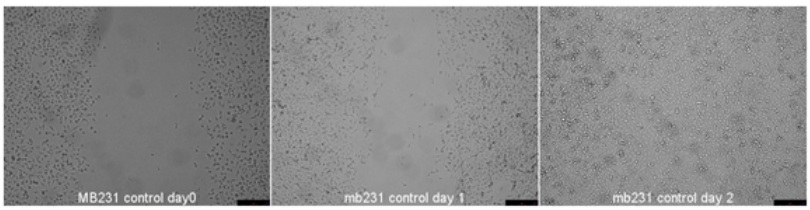

Fig. 1. Wound assay on control (untreated) MDA-MB-231 cells at 0-48h. Full wound healing at 48h is observed

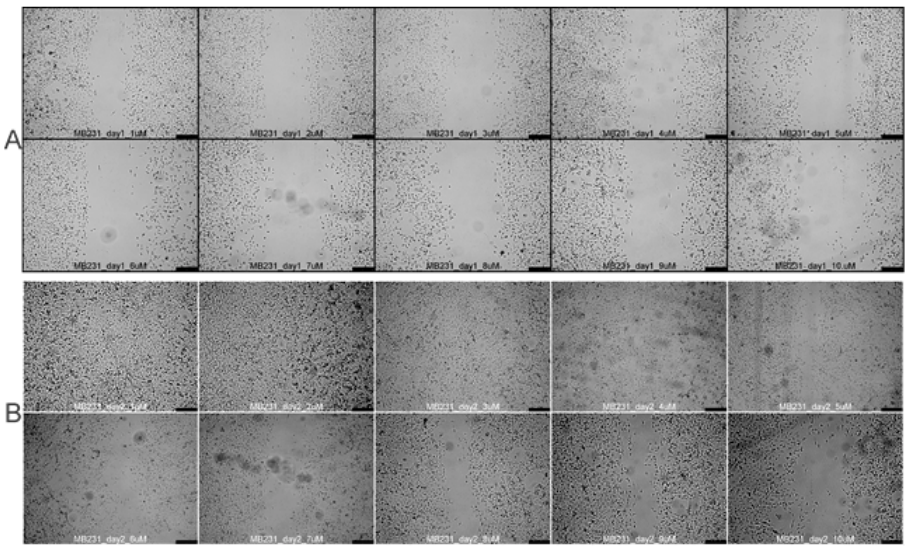

Fig. 2. Wound assay on MDA-MB-231 cells treated by curcumin in concentrations ranged from 1-10μM: A at 24h – single dose treatment; B at 48h – single dose treatment.

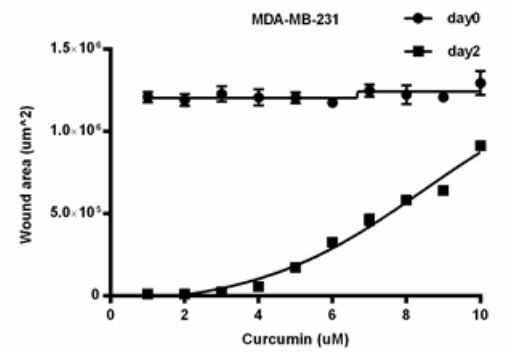

Fig. 3. Wound healing profile for MDA-MB-231 cells (lack of E-cadherin expression) following curcumin discontinuous treatment (single doses ranged from 1-10μM). At 48h, wound closure is almost complete for curcumin concentrations ranged from 1-4 μM). EC50 is recorded at curcumin concentration of 8.4 μM.

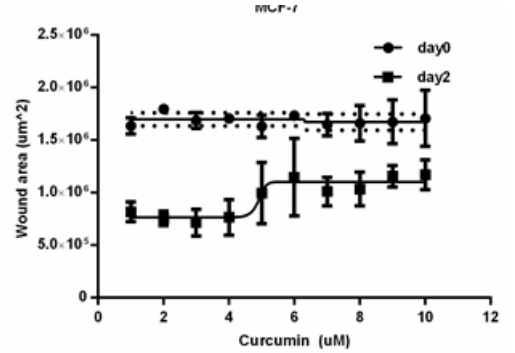

Fig. 4. Wound healing profile for MCF-7 cells (with positive E-cadherin expression) following curcumin discontinuous treatment (single doses ranged from 1-10 μM). At 48h, wound closure is almost 7 times lower than for MDA-MB-231 cells even for maximal curcumin dose applied in the assay. EC50 is recorded at curcumin concentration of 4.9 μM

MDA-MB-231 cells was noticed to be better for discontinuous curcumin treatment while for the continuous treatment (48+96h) the wound closure was impaired on more extended areas; this observation supports the curcumin involvement in reversing EMT, by inhibiting the invasive phenotype of these cancer cells. All observations were consistent either for discontinuous (48h) and continuous treatment (48+96h). For the discontinuous treatment and curcumin concentrations ranged from 1-4μM, the wound healing rate was close to controls (98.6 to 96.78%). Then, for each supplementary 1μM of curcumin concentration, the wound healing rate has been reduced by 10%, the process being consistent up to curcumin concentration of 9μM. For curcumin concentrations of 10μM, wound healing progressed up to only 36%. For the continuous treatment (48+96h) tumor cell response to curcumin was more important. Wound healing was limited to 82.19% for curcumin concentration of 5μM while for 10μM wound healing was reduced to only 27%.

Wound healing rate for MDA-MB-231 cells differed among discontinuous (48h) and continuous (48+96h) treatment and is displayed in figure 5. Differences between wound healing rates in the two depicted conditions are enhanced for curcumin concentrations ranged from 7-10μM. Continuous curcumin treatment inhibits better the invasive character for MDA-MB-231 cells.

At the end of migration assay we have also performed a viability assay to check the possibility that reduced cell migration can be influenced by curcumin-induced apoptotic cell death. Cell viability determined by Cell-Titer-

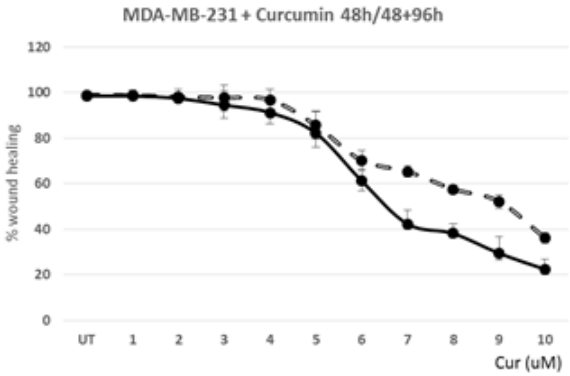

Fig. 5. Comparative profile of the percent wound healing on MDA-MB-231 cells for continuous versus discontinuous treatment by curcumin with concentrations ranged from 1-10 μM (UT – untreated sample)

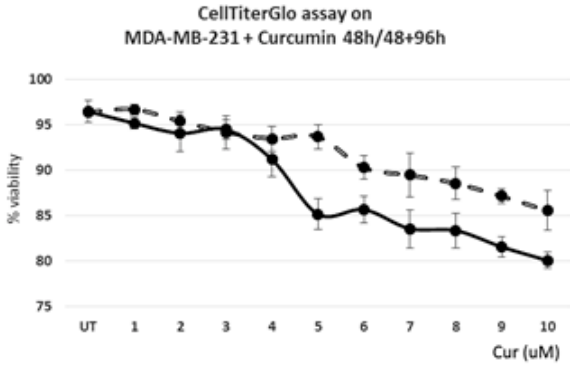

Fig. 6. Comparative viability ATP assay (CellTiterGlo) on MDA-MB-231 cells treated by continuous/discontinuous ranges of 1-10 μM Curcumin

Glo assay showed values between 80.1-97.2% for all curcumin dosage used, including the continuous treatment (fig. 6).

Curcumin ability to stimulate E-cadherin reactivation in MCF-7 cells submitted to mesenchymal character stimulation or cancer stem cells selection treatment was recently investigated and discussed [17, 18]. Thus, the present study is justified by the curcumin concentration range and the observed effect on mesenchymal and epithelial cell lines (as MDA-MB231 and MCF-7 respectively). The two cited papers show that a curcumin dose of 20-30μM induces cell proliferation arrest while in the present study the same effects were induced by just half the curcumin dose. At the same time, the cited studies are investigating the curcumin effect for just 24h while the present study used a curcumin pre-treatment with doses ranged from 1-10uM. In the cited papers, cell viability evaluation was performed by MTT assay which may show broad variations and poor specificity while the present investigation requires more accurate viability determination. Curcumin is able to resurrect the E-cadherin synthesis not only on MCF-7 cells submitted to EMT transition but also in MDA-MB-231 cell line, with natively blocked E-cadherin synthesis, by native gene hyper-=methylation. In cells that undergone EMT, E-cadherin expression is scarce while vimentin expression is augmented; vimentin is also a marker for full undifferentiated cell phenotype. Tumor cells expressing vimentin show a high proliferative and invasive potential [19-21]. Vimentin, as major intermediate filament in mesenchymal cells appear in early stages of the tumor progression and is regulated, together with E-cadherin, by Snail – a Zn-finger protein involved in transcription regulation; it is involved at the same time in embryo

mesoderm formation. During EMT processes, epithelial markers is diminished (E-cadherin, catenin α or β) while improved cell migration is associated with some mesenchymal markers as fibronectin vimentin, N-cadherin and α-SMA. Snail is binding and blocking the E-cadherin gene promoter by repressing its transcription. NF-kB is binding and regulating Snail gene promoter and is essential to EMT. NF-kB inhibition reduces Snail levels and induces the reverse EMT process. Curcumin and other antioxidative compounds inhibit LPS-induced or Snail (SNAI1) and Slug dependent EMT. Slug expression (protein that blocks E-cadherin expression and plays antiapoptotic roles) is inhibited by resveratrol [22]. Curcumin is also able to modulate Wnt/ β-catenin signaling pathways and can inhibit prostate carcinoma cells proliferation. Those two protein expression is influenced by Curcumin treatment in breast adenocarcinoma cells [23]. At the same time, Curcumin is able to interfere not only microtubule dynamics but also of the F-actin in breast and prostate carcinoma cells [24, 25].

Data regarding Curcumin toxicity against cells derived from breast adenocarcinomas have been published in over 100 papers in the past 15 years. Zhi-Dong Lv et al. [26] claim the curcumin effect onset on MDA-MB-231 or MCF-7 cell lines at 48h, with an IC50 of about 20μM. The cell viability profile for the two cell lines in the cited paper looks the same while in our results differ broadly in the present study. Thus, an accurate evaluation of curcumin toxicity in a dose-response manner on triple-negative breast cancer cells has not been yet performed. Moreover, in the mentioned study, cell viability was evaluated by MTT assay, not one of the most accurate available. Another study [27] describes the curcumin ability to inhibit cancer stem–like cells motility in breast carcinomas Here the maximal curcumin dose (15 μM) is close to the top limit in our study, but viability is evaluated by a less consistent assay (trypan blue). Loss of E-cadherin expression is a key mechanism for converting epithelial phenotype cells to metastatic phenotype. E-cadherin malfunctioning is associated to the β-catenin signaling and the loss of cooperation between the two proteins results in nuclear localization of the catenin [28, 29].

Curcumin seems to be involved in restoring cadherin-catenin interaction, avoiding nuclear transport of the β-catenin and also increases E-cadherin expression levels by Slug inhibition [30, 31].

Conclusions

The present study shows the low-dose curcumin effect on the migratory ability of triple negative breast cancer cells in culture. The results emphasize the differences between continuous and intermittent treatment of breast cancer cell lines with curcumin concentrations ranged from 1-10 μM. The best results, with consistent correspondence between migration ability inhibitions without reducing cell viability below 95% were obtained by a continuous treatment with 5 μM curcumin.

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