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Anticancer effects of curcumin in luminal B and HER2 breast cancer cell line models

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Abstract

In this study we aimed to test the activity of curcumin, a polyphenolic compound with known anti-cancer properties, in two breast cancer cell lines with different phenotypes: MCF-7 cell line as luminal B model and SK-BR-3 as HER2-amplified cancer cell model. As assayed by flow cytometric measurements 72 h curcumin treatment blocked the cell cycle progression of MCF-7 cells in G2/M phase, while for SK-BR-3 cells the cell cycle evolution was blocked less efficiently in the S phase. Curcumin reduced the clonogenic survival of both cell lines with EC_{50} values in the low micromolar range for the 48 h treatment period, SK-BR-3 being slightly more affected. In MCF-7 cells, curcumin reduced the expression level of ERK and its phosphorylation (Y204). Short term (0 to 120 min) treatment with curcumin increased the ROS production in both cell lines, but the effect was more pronounced in MCF-7 cells. In both cell lines, 48 h of 25 μM curcumin treatment induced mitochondrial membrane depolarization, with similar EC_{50} values. In conclusion, curcumin had anticancer effects in both cancer cell lines phenotypes, indicating its potential therapeutic usefulness against different types of breast cancer.

Keywords: curcumin, breast cancer cells, HER2, luminal B, clonogenic potential, mitochondrial membrane potential, reactive oxygen species

1. Introduction

Breast cancer is the most frequent malignancy in women world-wide, accounting for 23% of the total new cancer cases and is responsible of 14% of cancer deaths in this subpopulation (Jemal & al. [1]). Breast cancers as a whole have a relative low mortality rate (Ferlay & al. [2]) but they are also a highly heterogeneous group of diseases, with particular treatments and different prognosis. According to their extracellular receptors they can be divided in luminal A (ER+ and/or PR+, HER2- and Ki-67 low), luminal B (ER+ and/or PR+, HER2+/- and Ki-67 high), HER2-amplified and triple-negative (Yersal & al. [3]). The most frequent breast

cancer subtype, luminal A, accounting for 50-60% of all breast cancers, has a good prognosis and its treatment is mainly hormonal, the triple-negative phenotype accounts for about 17% of cases and has an intermediary prognosis, the worst prognosis being that of luminal B, and HER2-positive phenotypes, each accounting for about 15-20% of breast cancer cases (Cheang & al. [4, Yersal & Barutca & al. [3]).

The adjuvant therapy of luminal B cancer includes tamoxifen and endocrine treatment (Coates & al. [5]) while the treatment of HER2-positive breast cancers is centered on HER2 targeting, either by humanized monoclonal antibodies such as trastuzumab and pertuzumab, or by inhibiting the tyrosine kinase activity of the receptor by lapatinib, in combination with chemotherapy with paclitaxel or docetaxel. However, resistance to HER2 therapy often occurs and the treatment regimens using more therapeutic agents to overcome this resistance have severe side effects (Kumler & al. [6]).

Curcumin is a polyphenol from *Curcuma longa* studied for a plethora of effects including anti-infective (Mazumder & al. [7]), anti-inflammatory (Zhang & al. [8]), hepato- and cardiovascular protector (Fujise & al. [9, Kapakos & al. [10]), anti-trombotic (Kim & al. [11]) and anticancer. Among anticancer effects of curcumin, apoptosis inducement through the increased expression of death receptors 4 and 5 (Jung & al. [12, Yang & al. [13]), and decreased expression of a large number of kinases, enzymes and anti-apoptotic proteins such as Bcl-2 were shown (Shanmugam & al. [14]). Also, curcumin was shown to decrease HER2 levels and phosphorylation in cancer cells overexpressing this protein (Hong & al. [15]) through ubiquitination and degradation by carboxyl terminus of Hsc70-interacting protein (Jung & al. [16]).

As the many anticancer effects of curcumin were shown before in different cancer models, in this study we compared the effect of curcumin on models for the more aggressive forms of breast cancer, luminal B and HER2 overexpressing, using as models (Prat & al. [17]) the MCF-7 cell line, and HER2-amplified SK-BR-3, respectively.

2. Materials and methods

Cell cultures and treatments

Experiments were performed using mammary adenocarcinoma MCF-7 and SK-BR-3 cells from American Type Culture Collection. The culture medium was DMEM (Sigma) supplemented with 10% heat inactivated FBS (Sigma), 1% Penicillin/Streptomycin (Gibco), 2mM L-glutamine (Gibco) and, in the case of MCF-7, 10 µg/ml insulin. The cells were grown in a humidified incubator at 37°C, 5% CO_2. Adherent cells were detached by trypsinization and cells were washed in PBS before reseeding or use in experiments. Curcumin (Sigma) was used from fresh 50 mM stock solutions prepared in DMSO (Sigma) in the day of the experiment. In all control samples, the same volume of DMSO as in the treated samples was added.

Clonogenic assay

MCF-7 and SK-BR-3 cells (1000 and 2000 cells/well, respectively) were seeded in 6 well plates and let to adhere. The cells were treated for 48 h with 0 (control), 0.1, 0.5, 1, 2.5, 5, 10, 25, 50 and 100 µM curcumin in complete medium and incubated at 37 °C for 7 days, followed by 5 min fixation in 3.7% formaldehyde and 15 min staining with 0.5% crystal violet. The excess dye was washed with tap water. After drying, macroscopic pictures were taken. Colonies were counted automatically using the ImageJ v.1.48 software, EC50 values were calculated from inhibition curves fitted in GraphPad Prism v.5 using the following formula 1:

$$Y = Bottom + \frac{Top - Bottom}{1 + 10^{(LogEC50 - X) \times HillSlope}} \quad (1)$$

where X is logarithm with base 10 of the concentration of EGCG, LogEC50 is the logarithm with base 10 of the half-maximal effective concentration, Y is number of colonies normalized to control, "*top*" and "*bottom*" are plateau values.

Cell cycle evaluation

A number of 2 x 10^5 MCF-7 and SK-BR-3 cells were seeded in 6 well plates and let to adhere for 24 h. After that, the cells were synchronized in G0/G1 phase of the cell cycle by 24 h incubation in serum free media, then treated for 72 h with 0 (control), 5, 10, 25, 50, 100 µM curcumin, stained with propidium iodide in RNase staining buffer (BD Pharmingen) and measured with Gallios Beckman Coulter flow cytometer (excitation: 488 nm, emission: 620 ± 30 nm).

Intracellular staining of signaling molecules

Cells were plated in T75 flasks and let to adhere for 24 h and then treated with 0 (control) or 25 µM curcumin for 48 h. Trypsinized cells were aliquoted to 10^6 cells/sample, fixed in 3.7% paraformaldehyde (Sigma), washed in PBS, permeabilized with 0.2% Triton X-100 (PerkinElmer), washed and stained with 20 µl anti-ERK PE (Santa Cruz), 20 µg/ml monoclonal anti-pERK (E-4) FITC (Santa Cruz), antibodies for 1 h at 4 °C. The excess of the antibodies was removed during the final wash in PBS. The samples were measured with Gallios Beckman Coulter flow cytometer (excitation: 488 nm, emission: 525 ± 40 nm or excitation: 488 nm, emission: 575 ± 30 nm for FITC and PE, respectively).

Reactive oxygen species production assay

Cells were plated in 6 well plates at a concentration of 3-5x10^5 cells/well and let to adhere for 24 h and then treated with 25 µM curcumin for 0 (control), 10, 30, 60 and 120 min. Trypsinized cells were stained for 30 min with 5-(and-6)-carboxy-2′,7′-dichloro- dihydro-fluorescein diacetate (carboxy-H_2DCFDA, Molecular Probes) and washed in PBS; the samples were measured with Gallios Beckman Coulter flow cytometer (excitation: 488 nm, emission: 525 ± 40 nm).

Mitochondrial membrane potential assay

The MCF-7 and SK-BR-3 cells were grown in 6 well plates, incubated for 72 h with 0 (control), 5, 10, 25, 50 or 100 µM curcumin, stained 15 minutes with 5 µg/ml JC-1 (Thermo Fisher) at 37°C, trypsinized, washed in PBS and measured with Gallios Beckman Coulter flow cytometer (excitation: 488 nm, $emission_{green}$: 525 ± 40 nm, $emission_{orange}$: 575 ± 30 nm). Curcumin fluorescence was subtracted from treated, non-stained samples. EC50 values were calculated from inhibition curves fitted in GraphPad Prism v.5.

Data analysis

Data are represented as mean ± standard error of the mean (sem). Significance was assayed using unpaired t test or one way ANOVA with Bonferroni post-test for multiple column analysis. A value of $p < 0.05$ was set as level of significance. Raw data were analyzed using ImageJ v.1.48 (NIH, USA) and Flowing Software 2.5.1 (University of Turku, Finland). For statistical analysis, Microsoft Excel 2010 (Microsoft Inc., USA) and Prism 5 (GraphPad Software Inc., USA) were used.

3. Results and discussions

Curcumin inhibits the colony formation

In both MCF-7 and SK-BR-3 cell lines, 48 h curcumin treatment reduced the number of colonies in a dose dependent manner (Figure 1). For the more sensible to treatment cell line, SK-BR-3, curcumin concentrations of 25 µM and higher completely inhibited clonogenic growth, while in the MCF-7 cell line clonogenic growth was completely inhibited by concentrations higher than 50 µM. Thus, SK-BR-3 showed an EC_{50} value of 2.2 µM (best

fit, 95% confidence intervals from 1.08 to 4.49 μM), and MCF-7 showed an EC_{50} value of 12.3 μM (best fit, 95% confidence intervals from 5.06 to 29.74 μM). The overall lower concentrations needed for colony growth inhibition than those needed for other anticancer activities of curcumin shown in this study are in agreement with previous results showing lower curcumin concentrations needed for the inhibition of clonogenic formation than for the inhibition of proliferation as assayed in an MTT test (Hong & al. [15]). This might be explained by the fact that for a colony to reach macroscopic measurable sizes not only individual cells must survive but also they must proliferate. It was also shown before that in breast cancer cells oxidative stress hinders proliferation as NADPH is funneled from lipogenesis to ROS detoxification (Jerby & al. [18]). As we further show, curcumin blocks the evolution of the cell cycle and induces oxidative stress in both cell lines tested.

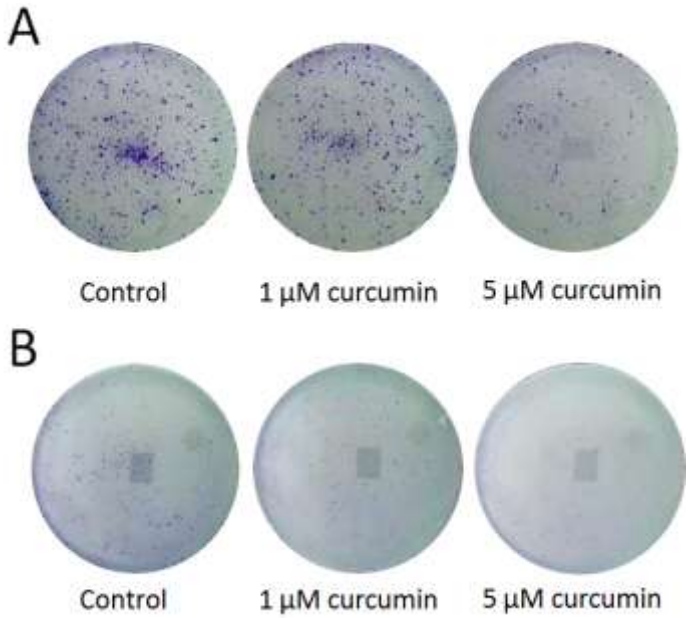

Figure 1. Curcumin reduces clonogenic potential of MCF-7- and SK-BR-3 cell lines. Representative images of curcumin treated MCF-7 (A) and SK-BR-3 (B) wells.

Curcumin affects cell cycle progression dependent on cell line

Curcumin blocked the cell cycle progression in different phases of the cell cycle depending of the cell line tested. Thus, in the MCF-7 cells, 25 μM curcumin treatment led to a reduction in G0/G1phase cells (from 70.1 ± 7 % to 48.3 ± 0.3 % of the viable cells), associated with an almost doubling in the number of cells in the G2/M phase (from 19.9 ± 4.9 % to 36.6 ± 3.7 %), as shown in Figures 2A and 2B. There is contradicting data on curcumin ability to block the cell cycle in luminal B cancer cells as some studies suggest that curcumin blocks MCF-7 cells in G2/M phase (Berrak & al. [19]) while others in G0 (Choudhuri & al. [20]), our data coming in support of the former theory. In SK-BR-3 cell line, the reduction in G0/G1 cell numbers was observed starting with the 10 μM curcumin treated sample (55.3 ± 1.1 % compared to 66.4 ± 0.3 %, unpaired t-test, $p<0.05$) associated with an increase in the S phase number of cells (22.1 ± 2.8 from 15 ± 0.03). The reduction in G0/G1 phase was maintained in the 25 μM curcumin treated sample (62.4 ± 0.4 % compared to 66.4 ± 0.3 %, unpaired t-test, $p<0.05$), in correlation with the increase of the number of cells in S phase (19.7 ± 1 from 15 ± 0.03, unpaired t-test, $p<0.05$), as shown in Figures 2C and 2D.

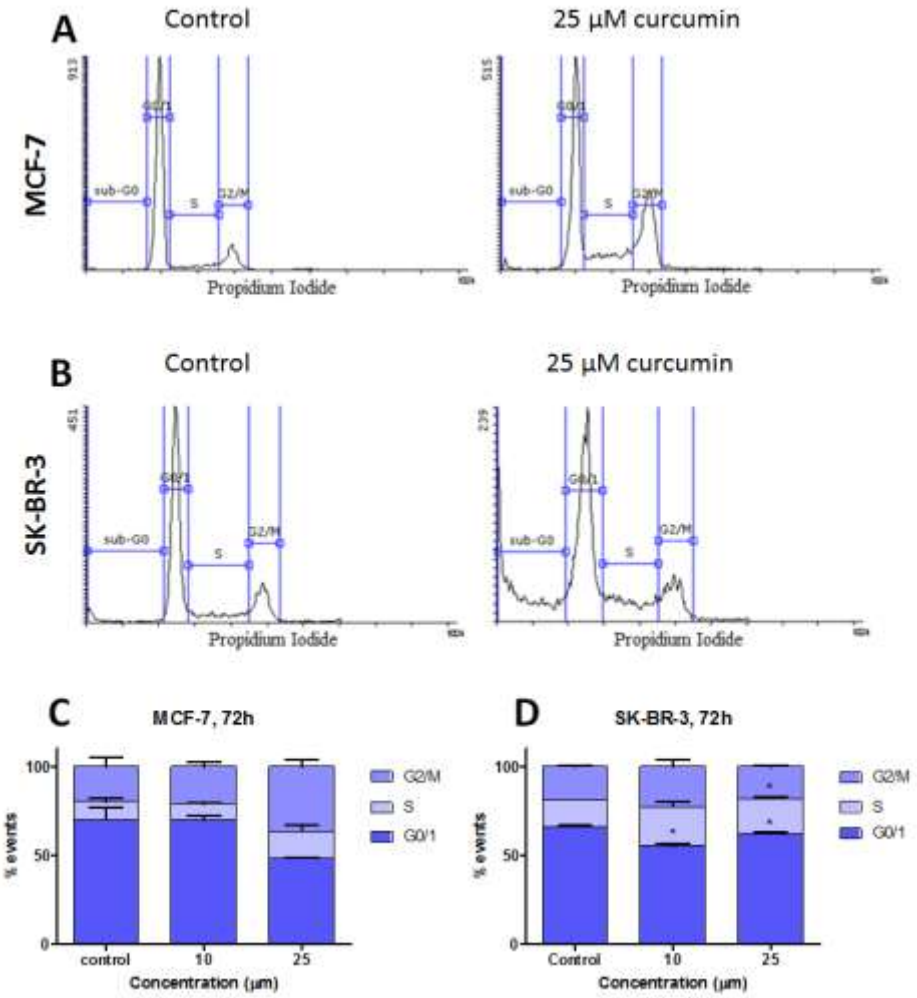

Figure 2. Curcumin blocks cell cycle evolution. Representative histograms for control and 25 µM curcumin treated MCF-7 (A) and SK-BR-3 (B) cells. (C) and (D) show barghraphs of percent cells in each cell cycle phase for the curcumin concentrations tested. *p<0.05.

Curcumin inhibits ERK expression and its phosphorylation at Y204 in cell line dependent manner

Extracellular signal regulated kinase (ERK) activation occurs downstream to insulin receptor as well as HER2 signaling and controls transcription factors, leading to increased proliferation and survival. Thus, we have been interested to see if the curcumin induced growth inhibition might be associated with a reduction in ERK expression or phosphorylation. Our experiments showed that in MCF-7 cells 48 h treatment with 25 µM curcumin greatly affects ERK expression (a threefold reduction in mean fluorescent intensity ratio, MFIR, from 53.3 ± 3.5 to 17.8 ± 0.1, unpaired t-test, $p<0.01$, Figure 3A) and, consequently, a drop in the phosphorylated form of ERK (pERK) was also observed (a MFIR reduction from 25 ± 2.3 to 13.2 ± 1.1, unpaired t-test, $p<0.05$). In contrast to this marked effect, in the SK-BR-3 cells the curcumin treatment had no effect in reducing ERK expression (MFIR for ERK was 24.8 ± 2.5 in control and 25.3 ± 7.3 in the 25 µM curcumin treatment condition) or phosphorylation (MFIR for pERK was 20.66 ± 0.7 in control and 20.2 ± 6 in the treatment condition, Figure 3B). As ERK is over activated in many cancers and plays a role in cell survival and proliferation (Boulton & al. [21]) they are a promising therapeutic target with specific

inhibitors being developed and patented (Samatar & al. [22]). A reduction in ERK expression in luminal B cancer cells by curcumin is likely to lead to a less aggressive form of disease.

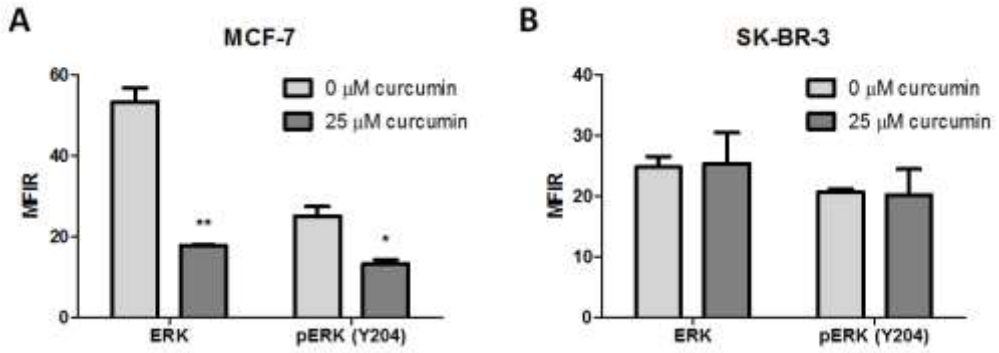

Figure 3. Curcumin can affect the expression of signaling molecules. Bargraphs showing ERK expression and tyrosine 204 phosphorylation reduction effect of curcumin in MCF-7 cell line (A) but no effect in the case of SK-BR-3 cell line (B) *p<0.05;**p<0.01.

Curcumin induces reactive oxygen species production

Next, we evaluated the ability of 25 μM curcumin to induce oxidative stress depending on the incubation time. Curcumin induced reactive oxygen species production in both cell lines in time dependent manner, MCF-7 cell line being more sensitive to this action (Figure 4). Thus, in this cell line curcumin induced a maximum 2.46 ± 0.18 fold increase in ROS production at 2 h (one-way ANOVA, p<0.001). In the SK-BR-3 cell line, ROS production reached a plateau after 1 h incubation at about 1.5 fold ROS production (1.55 ± 0.06 (one-way ANOVA, p<0.001) for the 1 h incubation and 1.57 ± 0.6 (one-way ANOVA, p<0.001) for the 2 h incubation). Rapid ROS production is perhaps the most important anti-cancer action of curcumin, being the initial signal in different pathways of apoptosis, both caspase-dependent and caspase-independent (Thayyullathil & al. [23]).

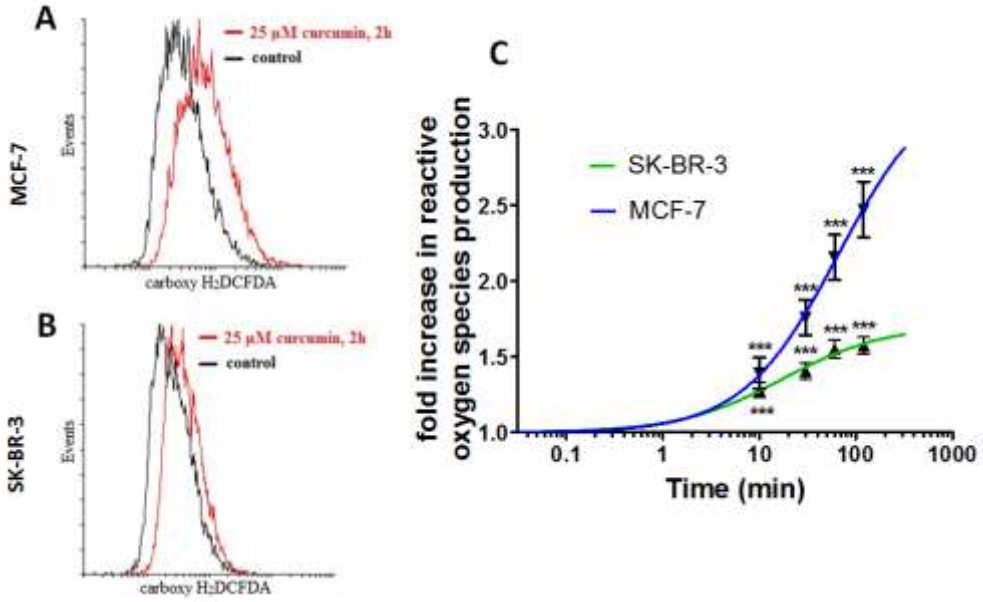

Figure 4. Curcumin induces reactive species formation dependent of the cell line. (A) and (B) show overlay histograms of 120 min vs. 0 min (control) 25 μM curcumin treated MCF-7 and SK-BR-3 cells. (C) The graph is showing treatment time dependence in the ROS production for the two cell lines.

Curcumin reduces the mitochondrial membrane potential (*ΔΨm*)

Mitochondrial membrane potential collapse is observed in all dead cells no matter the mechanism of this death: necrosis or apoptosis. However, in the case of apoptosis *ΔΨm* is affected early and thus, *ΔΨm* collapse before morphological changes is considered a hallmark of apoptosis (Lugli & al. [24]). To assay the death inducing actions of curcumin we tested the *ΔΨm* after short (0-120 minutes) and long (48 h) treatment incubation periods. In the short term incubation, even if curcumin seemed to increase the percent of cells with depolarized mitochondrial membranes, the effect wasn't strong enough to reach statistical significance (Figure 5).

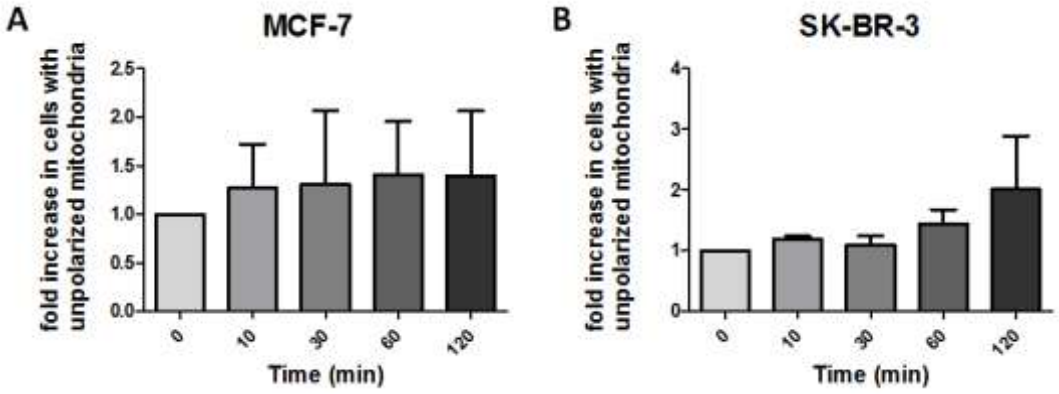

Figure 5. Up to 120 minutes incubation with 25 μM curcumin did not significantly induce *ΔΨm* colapse. Shown are bargraph of fold increase in MCF-7 (A) or SK-BR-3 (B) cells with depolarized mitochondrial membranes at different time points after 25 μM curcumin treatment.

When *ΔΨm* was assayed after 48 h treatment, curcumin induced mitochondrial membrane potential collapse in dose dependent manner in both cell lines (Figure 6), with similar efficiencies. In the MCF-7 cell line the EC_{50} value was 44.1 μM (best fit, 95% confidence interval from 42.40 to 45.94 μM) and in the SK-BR-3 the EC_{50} value was 34.7 μM (best fit, 95% confidence interval from 25.3 to 47.7 μM).

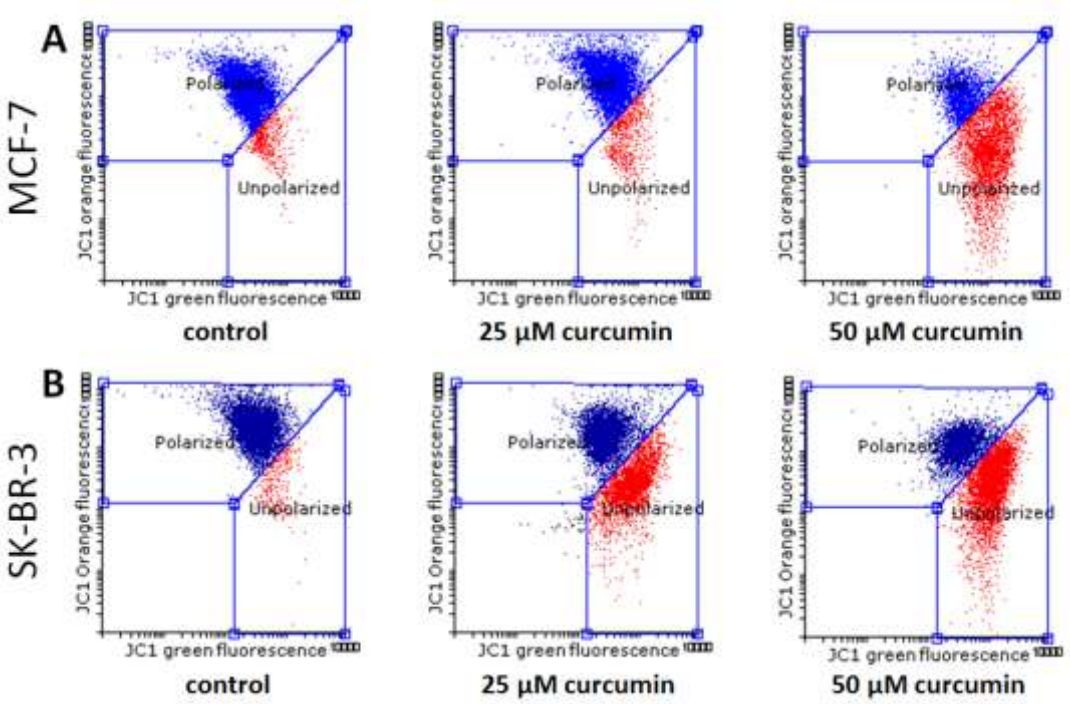

Figure 6. Curcumin treatment for 48h reduces the mitochondrial membrane potential. shows dot-plots of JC1 green vs. orange fluorescence in (A) MCF-7, (B) SK-BR-3 cells treated with 0, 25 or 50 µM curcumin. The cells with more JC1 aggregates, emitting in orange, have normal polarized mitochondrial membranes, while those with JC1 mainly in monomeric form, emitting in green, have depolarized mitochondrial membranes.

4. Conclusions

As shown here, curcumin treatment leads to similar outcomes in the luminal B and HER2 amplified cell lines tested, with similar curcumin concentrations required for mitochondrial membrane depolarization, with oxidative stress induction and inhibition of clonogenic formation in both models but with slightly different sensitivities. Curcumin also presented cell line specific effects, blocking the cell cycle evolution in different stages – G2/M phase for luminal B MCF-7 and S phase for HER2-amplified SK-BR-3 – and reducing the expression and Y204 phosphorylation of ERK only in the luminal B model. The results presented here provide insights in the anti-cancer effects of curcumin in different breast cancer models and could offer some use in developing new treatment strategies.

5. Acknowledgements

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