

## ***In vitro* evaluation of curcumin effects on breast adenocarcinoma 2D and 3D cell cultures**

HUSSAM ABUELBA<sup>1)</sup>, CARMEN ELENA COTRUTZ<sup>1)</sup>, BOGDAN ALEXANDRU STOICA<sup>2)</sup>,  
 LAURA STOICA<sup>1)</sup>, DOINIȚA OLINICI<sup>1)</sup>, TUDOR PETREUȘ<sup>1)</sup>

<sup>1)</sup>Department of Cell and Molecular Biology, "Grigore T. Popa" University of Medicine and Pharmacy, Iassy, Romania

<sup>2)</sup>Department of Biochemistry, "Grigore T. Popa" University of Medicine and Pharmacy, Iassy, Romania

### **Abstract**

Breast adenocarcinoma cell line MDA-MB-231, even if it expresses low levels of E-cadherin, still readily form multicellular aggregates of cells, namely spheroids. Curcumin is a diarylheptanoid antitumoral drug while it significantly inhibits cell migration, invasion, and colony formation *in vitro* and reduces tumor growth and liver metastasis *in vivo*. Curcumin photoactivation may enhance antiapoptotic role against cancer cells. *Aim:* To evaluate the effect of low curcumin concentrations, ranged from 1.9 to 15  $\mu$ M, with and without photoactivation, using a manufactured 670 nm LED-matrix. A secondary aim was to evaluate the ideal method to produce easy-to-use tumor cell spheroids, comparing two low adherence plate supports. *Materials and Methods:* Breast adenocarcinoma cell line MDA-MB-231 were cultured according to 2D monolayer and 3D spheroid models then submitted to normal and photoactivated curcumin in micromolar concentrations. MTT assay was used to evaluate cell viability following curcumin application on cells. On 2D cell cultures, curcumin inhibits cell tumor development and proliferation at concentrations of 15  $\mu$ M, with a viability of 65.7% at 48 hours incubation time. A decreased viability up to 25% for a concentration of 15  $\mu$ M was recorded following photoactivation and cytotoxic action on breast cancer tumor cell line continued at concentrations of 7.5 and 3.75  $\mu$ M. Curcumin photoactivation increases pro-apoptotic effects in both 2D and 3D tumor cell culture models and also responsiveness to curcumin is slightly reduced in spheroid-like structures. Thus, 3D tumor cell culture systems appear to be the ideal environment for *in vitro* assays regarding anticancer drug effects on cell viability.

**Keywords:** spheroids, invasive ductal carcinoma, curcumin, photoactivation.

### **Introduction**

Most mechanical interactions in epithelial tissues are based on cell-to-cell adhesion molecules, and mainly on cadherins (E-cadherins and desmosomal cadherins). E-cadherins represent a part of cadherin family glycoproteins and are being involved in  $\text{Ca}^{2+}$ -dependent cell-to-cell interactions, playing an important role in the maintenance of epithelia integrity. Reduced cell adhesion is one of the main features of cancer cells, lack inhibition contact leading to tumor growth, invasive character and cell detachment able to produce metastases. Literature data advocate that many cancers [1–3] (hepatocellular, gastric, colon, lung, breast) show abnormal expression of E-cadherins [4–6]. Reduced E-cadherin expression was found to be correlated with higher tumor staging and degree of dedifferentiation, leading usually to metastasis and lower survival rates. Curcumin, one of the major natural anticancer agents and the main constituent in the curry spice turmeric, was crystallized in 1870 while its crystallographic structure was defined in the early 20th century. Curcumin was found to be useful in the treatment of various cancer forms by inhibiting cell proliferation, angiogenesis and metastasis. It is also recognized as anti-inflammatory, antioxidant, antimicrobial and wound healing promoting agent. These effects are usually due to interactions between curcumin and multiple cell-surface receptors or cytokines. Curcumin is also stimulating tumor cell apoptosis at a far more important rate than in

normal cells, thus being an important advantage over most chemotherapeutic agents. Despite anti-proliferative and apoptosis inducing effects on various tumor cells at concentrations ranging from 10 to 150  $\mu$ M, curcumin shows low oral bioavailability and low solubility in physiological fluids.

The aim of the present study was to explore the effect of low curcumin concentrations, ranged from 1.9 to 15  $\mu$ M, with and without photoactivation, using a manufactured 670 nm LED-matrix. A secondary aim was to evaluate the ideal method to produce easy-to-use tumor cell spheroids from triple negative breast tumor cells, comparing two low adherence plate supports.

### **Materials and Methods**

#### **Cell culture**

Breast adenocarcinoma cell line MDA-MB-231 (CLS, Germany) that are supposed to form aggregates or spheroids, being extensively studied in cancer research applications, were cultivated in DMEM media supplemented with 10% fetal calf serum and 1% Penicillin–Streptomycin solution (10 000 IU/mL Penicillin; 10 000 mg/mL Streptomycin). Cell cultures were maintained in 2D monolayers in a humidified incubator at 37°C, 5%  $\text{CO}_2$ .

#### **2D cultures – in monolayer**

At passage 9, cells were detached by a usual trypsin

protocol, counted and distributed in 96-well plates for MTT assay at a concentration of  $1 \times 10^5$  cells/well. MDA-MB-231 cells were co-incubated with four curcumin micromolar concentrations as described below. At 48 hours, culture media was gently removed from each well avoiding cell layer scratch, wells were washed by 100  $\mu$ L PBS (phosphate-buffered saline) and then MTT assay was performed as described below.

### 3D cultures – spheroid formation

MDA-MB-231 cells at 9<sup>th</sup> passage were transferred as cell suspensions in agarose-coated 96-well plates. Prior cell transfer, gel coating was prepared from a 1.5% (*wt/vol*) agarose solution obtained by combining 0.15 g agarose (Sigma-Aldrich, EU) with 10 mL PBS. By micro-waving, agarose was dissolved in about 30 seconds and kept on a hot plate at 85°C during the well coating process, to avoid premature gelation. Well coating was performed by 70 mL hot agarose solution pipetted into each well by heated tips and allowed to cool for 20 minutes. Cell concentration was chosen by sequential assays on  $1 \times 10^4$  and  $1 \times 10^5$  cells/well. Those concentrations demonstrated good ability to form spheroids in agarose coated 96-well plates. For each well, cells were suspended in 100  $\mu$ L DMEM.

### MTT assay

Cell viability by MTT assay [7] is a versatile method used to evaluate the cell survival based on a colorimetric method that uses a tetrazolium salt (MTT), which is transformed by mitochondrial dehydrogenases in purple formazan granules. Following medium removal and PBS washing, cells were incubated with MTT solution (1 mg/mL in PBS) for three hours and the resulting formazan was dissolved by DMSO (100  $\mu$ L). Absorbance was measured at 590 nm using an automated multiplate reader (Pharmacia LKB Ultrospec Plus). Cell viability was expressed as percent compared to control lanes (blank – culture medium without cells; control – culture medium with cells) according to the formula  $CV = 100 \times (OD_s - ODb) / (ODc - ODb)$ , where  $OD_s$  – the optical density (in units) for the sample,  $ODb$  – the optical density for the blank wells, and  $ODc$  – the optical density for the control wells. Assays for

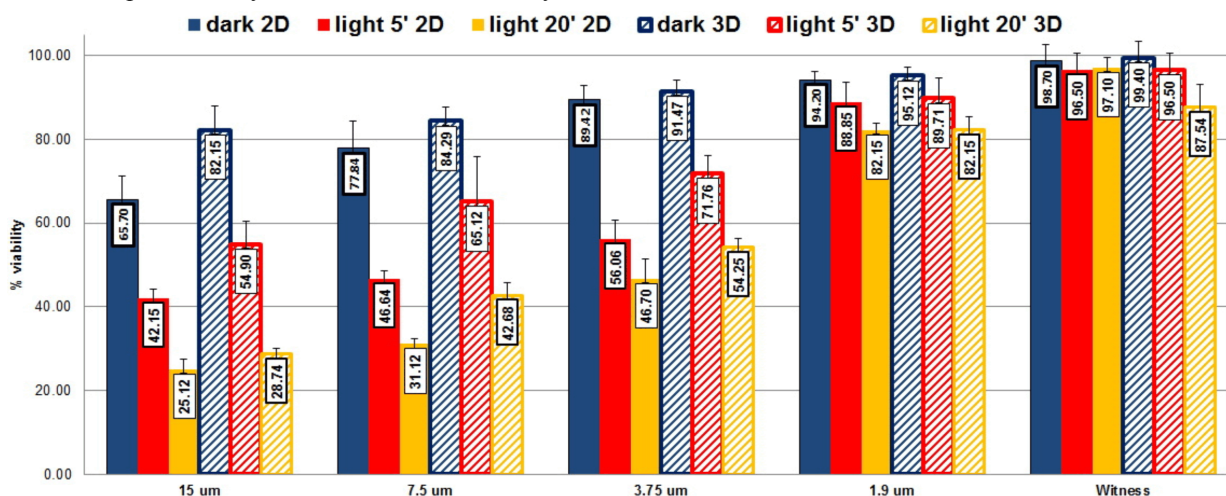
each extract were carried out in three replicates, including untreated cell control and the blank cell-free control.

### Cell/well concentration and photoactivation experiment design

Curcumin powder was dissolved as 1.1 mg in 1 mL DMSO. From this stock solution, 60  $\mu$ L were diluted in 5940  $\mu$ L DMEM to reach a concentration of 30  $\mu$ M. Then serial dilutions were performed in DMEM (15  $\mu$ M, 7.5  $\mu$ M and 3.5  $\mu$ M respectively); while in each well of the 96-well plate, 100  $\mu$ L from serial dilutions were placed and another 100  $\mu$ L  $\alpha$ MEM were added, the final curcumin concentrations were defined as C1=15  $\mu$ M; C2=7.5  $\mu$ M; C3=3.75  $\mu$ M and C4=1.9  $\mu$ M respectively. Each concentration was applied in triplicate. Witness wells included only cells in 200  $\mu$ L DMEM. Three 96-well plates were used for this viability assay. One plate was wrapped in sterile aluminum foil to be protected from reflected light during curcumin photoactivation. The other 96-well plate (destined to curcumin photoactivation) was placed on a sterile manufactured wireless LED matrix, each led being precisely focused on each well and covered by a mirror. Photoactivation was performed for two of the three plates, for 5 and 20 minutes respectively. LED matrix was slightly heated during usage, but not over 37°C. Plates were imaged at 24, 48 and 72 hours for 3D cultures with a Nikon D-5000 camera adapted by a C-tube to a Nikon T-3000 reversed phase contrast microscope.

### Results

MTT viability assays performed on 2D cell cultures showed that curcumin inhibits cell tumor development and proliferation at concentrations of 15  $\mu$ M, with a viability of 65.7% at 48 hours incubation time. Curcumin had less pro-apoptotic effects on MDA-MB-231 cells grown in monolayer with no photoactivation. However, light effects on curcumin activation were impressive for 20 minutes light activation time, with a viability decrease up to 25% for a concentration of 15  $\mu$ M. At the same time, light-activated curcumin remained active on breast cancer tumor cell line at concentrations of 7.5 and 3.75  $\mu$ M, respectively (Figure 1).

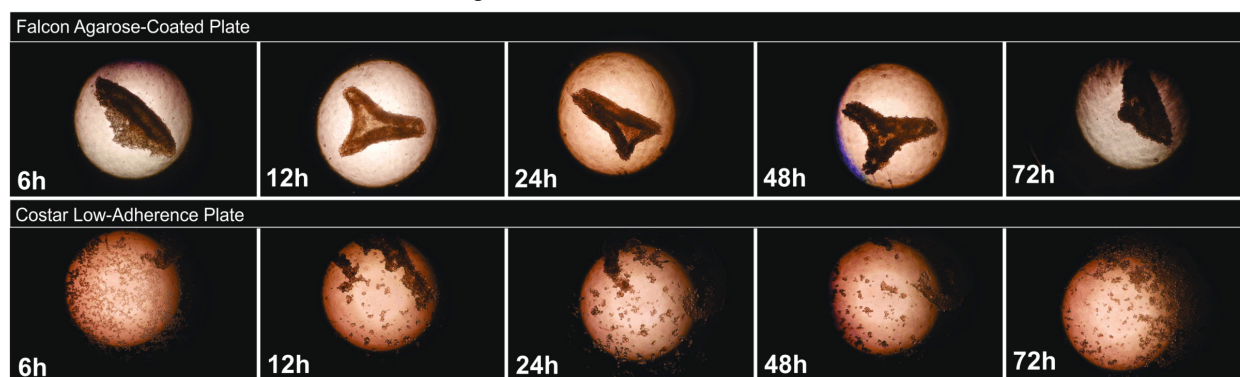


**Figure 1 – MTT assay evaluating cell viability following incubation with normal (dark) and photoactivated (light for 5 and 20 minutes) curcumin, in 2D and 3D spheroid cell cultures. Curcumin concentrations ranged from 15  $\mu$ M to 1.9  $\mu$ M by seriated dilutions.**

These effects were similar but with a reduced amplitude also on 3D cultures. These results show the reproducibility of these transitions from 2D to 3D models for curcumin effects on tumor cells. The cell viability in 3D spheroids was slightly increased compared to 2D monolayers for the same experimental conditions (curcumin incubation at 48 hours, without/with light exposure at 5 and 20 minutes and at cell amount of  $1 \times 10^5$ /well in a 96-well plate). Reduced viability in 3D cultures in the witness wells may be due to the trypsin treatment required to dismantle spheroids prior to viability assay procedure. However, in Figure 1, the curcumin effects on 3D culture are almost reaching the values

obtained in 2D cultures following photoactivation; we may then conclude that photoactivation leads not only to a dose dependent effect comparable with the effect on 2D cultures but also is reducing the required concentration to reach cytotoxic effects.

Another aim of the present research was to compare MDA-MB-231 spheroid formation ability in special low-adherence plates and agarose coated plates. Despite the producer details for low adherence well plates, we have obtained no single spheroid while spreading MDA-MB-231 cells compared to agarose-coated wells in normal 96-well culture plates (Figure 2).



**Figure 2 – Comparison between agarose-coated and low-adherence plates for spheroid formation from MDA-MB-231 breast cancer cells.**

At three days from cell spreading (at a concentration of  $1 \times 10^5$  cells/well in 96-well plates (low-adherence and agarose), very few small cell clumps were formed in low-adherence well plates while in agarose coated wells, cells formed spheroid-like agglomerations. Thus, the MDA-MB-231 cells viability assay following incubation with various concentration of curcumin was further performed only on 96-well plates coated by agarose as described in materials and methods.

Prior curcumin application on MDA-MB-231 cell spheroids, various cell concentrations/well were evaluated. The optimal cell amount per well in a 96-well plate appeared to be ranged from  $1 \times 10^4$  and  $1 \times 10^5$ . Two 96-well plates were filled, in different lanes, with two different cell amounts ( $1 \times 10^4$  and  $1 \times 10^5$ ) respectively and co-incubated with the defined curcumin concentrations (C1 to C4 as previously described).

The results following MDA-MB-231 cells incubation with various concentrations of curcumin showed a clear influence on cell viability for photoactivated curcumin. Not only cells underwent important apoptosis at  $15 \mu\text{M}$  but also cell viability and aggregation in 3D spheroids was affected following photoactivation. Viability (expressed by the ability of tumor cells to adhere and form 3D spheroids) for both cell concentrations was similar. At the same time, curcumin photoactivation effects were time dependent. Thus, a 20 minutes photoactivation led to visible enhancement of pro-apoptotic/cytotoxic effects for a curcumin concentration of  $7.5 \mu\text{M}$  and even for  $3.75 \mu\text{M}$ , compared to an only five minutes of photoactivation. The effects were more visible for cell amounts of  $1 \times 10^5$ /well (Figure 3).

Curcumin effects continued to develop also at 48 and 72 hours from incubation and photoactivation (Figures 4

and 5). The cytotoxic curcumin effects on tumor cells continued to be important at concentrations of  $7.5$  and  $3.75 \mu\text{M}$  for a 20 minutes photoactivation. Differences in spheroid formation are due not only to various degrees of cell survival but also to direct curcumin effect on cell adhesion.

## Discussion

While epithelial and myoepithelial cells in the normal mammary gland are well organized in acini and ducts and separated by a basement membrane from the extracellular matrix (ECM) in the stroma, cells in the invasive breast carcinoma directly contacts stromal cells, endothelium of newly form blood vessels, white blood cells, fibroblasts, as well as various compounds of the ECM [8]. A special microenvironment is developed around tumor cell clusters, and cell behavior, phenotype and destiny depend directly on the interactions among all cells and soluble factors in this mixture.

Most cytotoxicity assays destined to evaluate anticancer drug effects on tumor cells are using classic 2D cultures on plastic wells. Thus, cell-to-cell interactions in monolayer cultures are altered while morphological architecture and tissue functions are usually lost. Due to lack of cell signaling in direct contact with ECM, many cell characteristics that are available in tumor microenvironment are being lost in 2D cultures [9, 10]. This is the reason why three dimensional *in vitro* cell culture models are more predictive than monolayers in 2D cultures [11, 12]. Most 3D culture models are supposing tumor cell seeding on polymer scaffolds or cell embedding in hydrogels [11, 13] while some models are speculating adherent cell ability to cluster in suspension or on low adherence surface. While cell behavior in cell clusters is essential to further



development of tumor microenvironment, the present work tried to emphasize morphological changes that may appear during curcumin treatment of breast tumor cell spheroids. While curcumin effects on tumor cells are well documented in 2D environments, few studies approached curcumin effects in 3D cultures [14]. At the same time, photoactivated curcumin effects on tumor cells is documented mainly in 2D *in vitro* models and not in 3D cultures [15]. Curcumin, a traditional Asian spice extract, plays antitumor, anti-inflammatory, antioxidant and antimicrobial effects, and some of these effects are enhanced by photoactivation [16, 17]. Curcumin photochemistry

advocated that the triplet-excited states of curcumin may absorb light at 720 nm and may produce singlet molecular oxygen species in contact with oxygen [18]. The present work used a white bright LED matrix to activate curcumin, considering also that curcumin photodegradation may generate also smaller phenols, also active on tumor cells [19]. Considering the curcumin effects on tumor cells and apoptosis induction that is well documented in 2D and 3D cultures [20, 21], the present study demonstrated important differences in dynamic cell behavior in 3D spheroids submitted to normal/photoactivated curcumin action.

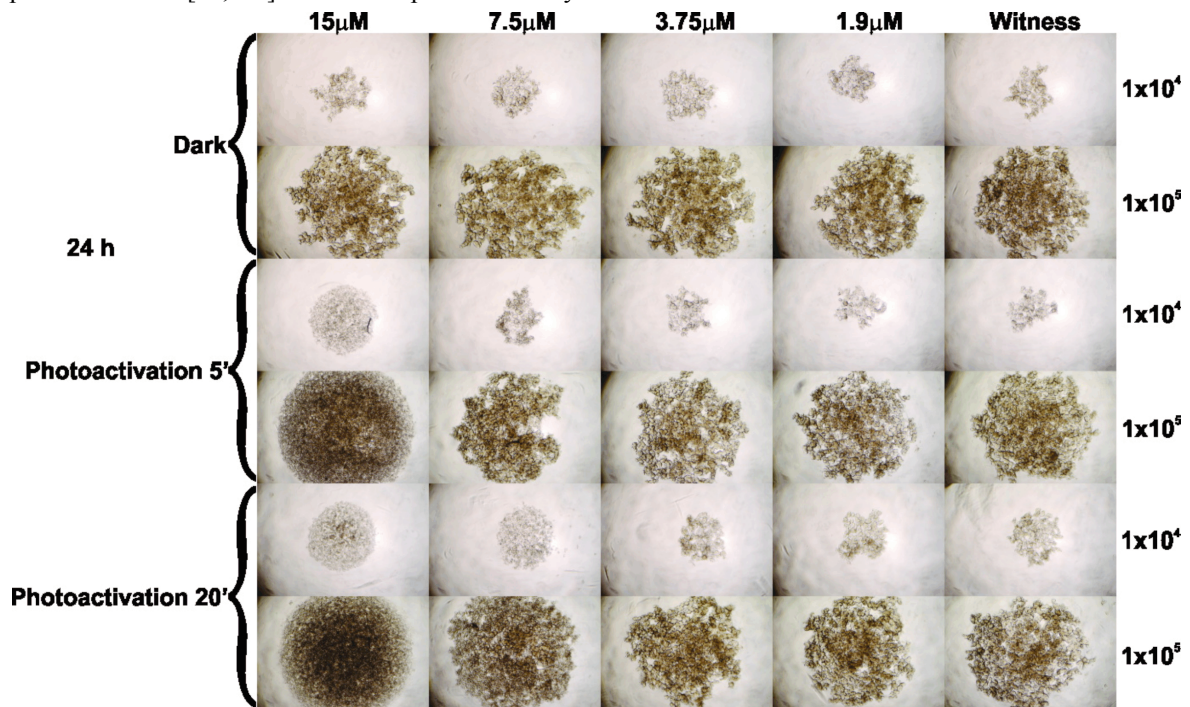


Figure 3 – MDA-MB-231 spheroid behavior at 24 hours incubation time with normal (dark) and light-activated curcumin (for 5 and 20 minutes).

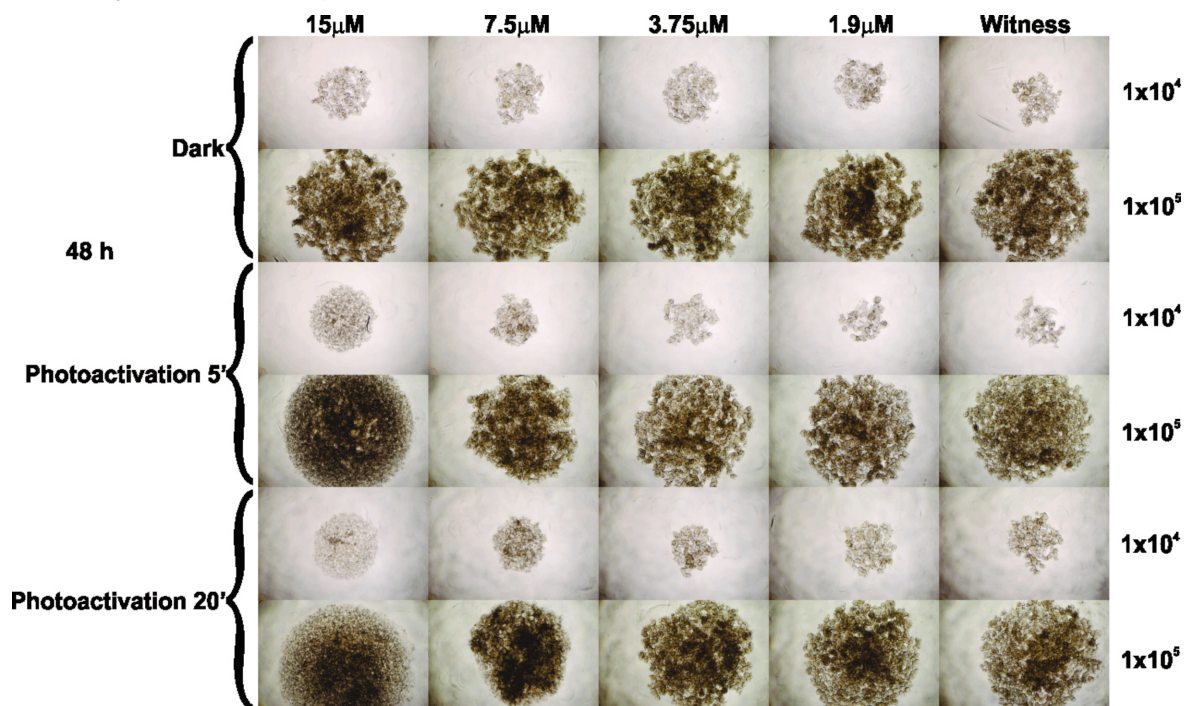
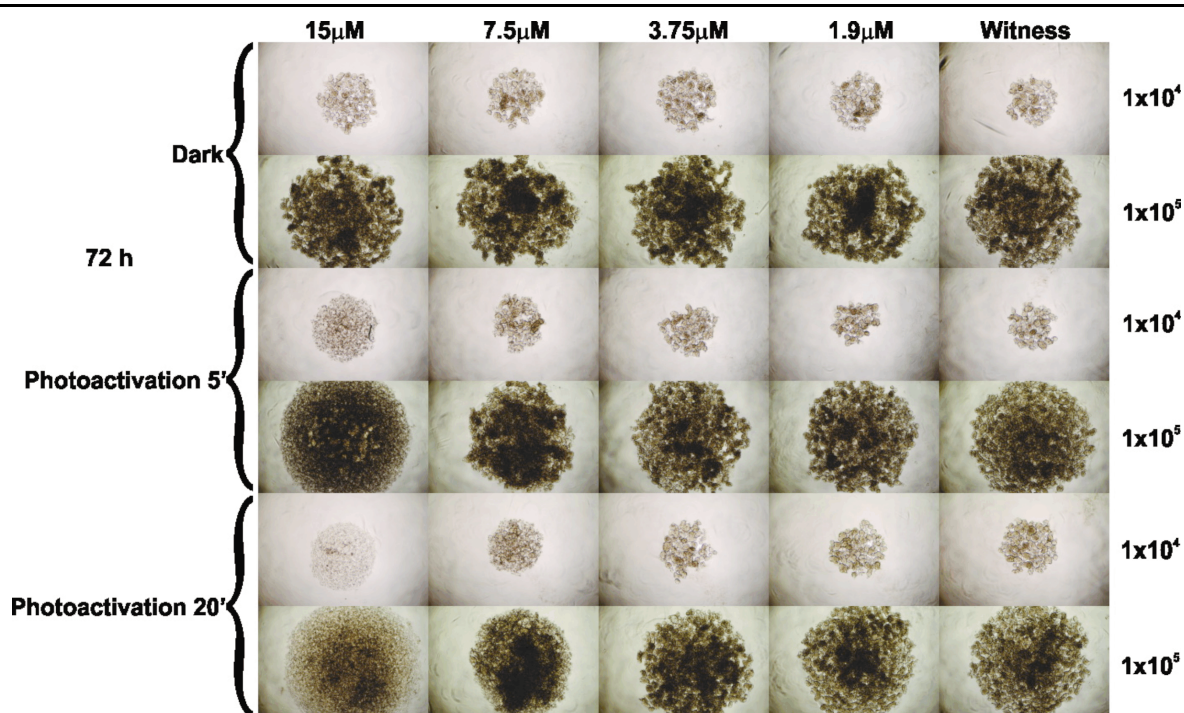


Figure 4 – MDA-MB-231 spheroid behavior at 48 hours incubation time with normal (dark) and light-activated curcumin (for 5 and 20 minutes).



**Figure 5 – MDA-MB-231 spheroid behavior at 72 hours incubation time with normal (dark) and light-activated curcumin (for 5 and 20 minutes).**

Cell viability evaluated by MTT assay following 2D and 3D cultures of MDA-MB-231 cells incubation with normal/light-activated curcumin in various lower concentrations showed similar results; in 2D cultures, viability was slightly lower than in 3D spheroid cultures probably due to cell clustering effect in 3D cultures that may avoid curcumin penetration over central cells in the spheroid. However, cell viability in witness 3D cell culture was slightly lower (87.54% compared to 97.10% in 2D monolayer) probably due to pro-apoptotic effect of cell clustering, combined with trypsin treatment of cell spheroids prior to viability assay.

Moreover, morphological differences were noted among various light exposures for curcumin. Thus, a 20 minutes exposure to LED bright light have enhanced antitumor effect even at lower curcumin concentrations up to 7.5  $\mu\text{M}$  and even at 3.75  $\mu\text{M}$ . These results were reproduced not only at 24 hours of incubation but also at 72 hours, even if the effects for a concentration of 3.75  $\mu\text{M}$  were slightly diminished. These photoactivated curcumin effects on tumor cells that have developed in spheroid 3D cultures should be further evaluated in respect to cadherin expression and cadherin gene alterations in tumor-simulated microenvironment. It was demonstrated that E-cadherin expression in colorectal carcinoma cell culture, together with other cell surface markers involved in invasiveness, are being inhibited by curcumin [21]. Spheroid analysis in further migration assays and microarray analysis is a point to be reached in current research on curcumin effects on 3D tumor cell cultures.

## Conclusions

Curcumin photoactivation increases drug ability to induce cell death in both 2D and 3D tumor cell culture models and also responsiveness to curcumin is slightly reduced in spheroid-like structures. However, at 15  $\mu\text{M}$

curcumin concentration, effects in 2D and 3D breast carcinoma cell cultures are impressive following light activation and in close ranges one to another. Curcumin photoactivation leads not only to a dose dependent effect comparable with the effect on 2D cultures but also is reducing the required concentration to reach cytotoxic effects. Thus, 3D tumor cell culture systems appear to be the ideal environment for *in vitro* assays regarding anticancer drug effects on cell viability. Also, to develop easy-to-use 3D spheroid based cell culture models is better to adopt faster and feasible methods as growing cell suspensions in agarose-coated 96-well plates, avoiding contamination risk of hanging drop procedure or cell seeding on polymer scaffolds/cell embedding in hydrogels that are expensive and time consuming.

## Conflict of interests

The authors declare that they have no conflict of interests.

## References

- [1] Shiozaki H, Oka H, Inoue M, Tamura S, Monden M. E-cadherin mediated adhesion system in cancer cells. *Cancer*, 1996, 77(8 Suppl):1605–1613.
- [2] van Roy F. Beyond E-cadherin: roles of other cadherin superfamily members in cancer. *Nat Rev Cancer*, 2014, 14(2):121–134.
- [3] Canel M, Serrels A, Frame MC, Brunton VG. E-cadherin–integrin crosstalk in cancer invasion and metastasis. *J Cell Sci*, 2013, 126(Pt 2):393–401.
- [4] Guilford P, Hopkins J, Haraway J, McLeod M, McLeod N, Harawira P, Taite H, Scoular R, Miller A, Reeve AE. E-cadherin germline mutations in familial gastric cancer. *Nature*, 1998, 392(6674):402–405.
- [5] Hajra KM, Chen DY, Fearon ER. The SLUG zinc-finger protein represses E-cadherin in breast cancer. *Cancer Res*, 2002, 62(6):1613–1618.
- [6] Coppola V, Bonci D. A tight junction between E-cadherin and the prostate tumor suppressor SPDEF. *Asian J Androl*, 2013, 15(4):449–450.



- [7] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods*, 1983, 65(1–2):55–63.
- [8] Weigelt B, Ghajar CM, Bissell MJ. The need for complex 3D culture models to unravel novel pathways and identify accurate biomarkers in breast cancer. *Adv Drug Deliv Rev*, 2014, 69–70:42–51.
- [9] Brown BN, Badylak SF. Extracellular matrix as an inductive scaffold for functional tissue reconstruction. *Transl Res*, 2014, 163(4):268–285.
- [10] Werfel J, Krause S, Bischof AG, Mannix RJ, Tobin H, Bar-Yam Y, Bellin RM, Ingber DE. How changes in extracellular matrix mechanics and gene expression variability might combine to drive cancer progression. *PLoS One*, 2013, 8(10):e76122.
- [11] Charoen KM, Fallica B, Colson YL, Zaman MH, Grinstaff MW. Embedded multicellular spheroids as a biomimetic 3D cancer model for evaluating drug and drug-device combinations. *Biomaterials*, 2014, 35(7):2264–2271.
- [12] Huttmacher DW. Biomaterials offer cancer research the third dimension. *Nat Mater*, 2010, 9(2):90–93.
- [13] Fallica B, Makin G, Zaman MH. Bioengineering approaches to study multidrug resistance in tumor cells. *Integr Biol (Camb)*, 2011, 3(5):529–539.
- [14] Shakibaei M, Buhrmann C, Kraeche P, Shayan P, Lueders C, Goel A. Curcumin chemosensitizes 5-fluorouracil resistant MMR-deficient human colon cancer cells in high density cultures. *PLoS One*, 2014, 9(1):e85397.
- [15] Balaji B, Somyajit K, Banik B, Nagaraju G, Chakravarty AR. Photoactivated DNA cleavage and anticancer activity of oxovanadium(IV) complexes of curcumin. *Inorg Chim Acta*, 2013, 400:142–150.
- [16] Sharma RA, Gescher AJ, Steward WP. Curcumin: the story so far. *Eur J Cancer*, 2005, 41(13):1955–1968.
- [17] Wood S, Nattress B, Kirkham J, Shore R, Brookes S, Griffiths J, Robinson C. An *in vitro* study of the use of photodynamic therapy for the treatment of natural oral plaque biofilms formed *in vivo*. *J Photochem Photobiol B*, 1999, 50(1):1–7.
- [18] Priyadarsini KI. Photophysics, photochemistry and photobiology of curcumin: studies from organic solutions, biomimetics and living cells. *J Photochem Photobiol C Photochem Rev*, 2009, 10(2):81–95.
- [19] Andrade MC, Ribeiro AP, Dovigo LN, Brunetti IL, Giampaolo ET, Bagnato VS, Pavarina AC. Effect of different pre-irradiation times on curcumin-mediated photodynamic therapy against planktonic cultures and biofilms of *Candida* spp. *Arch Oral Biol*, 2013, 58(2):200–210.
- [20] Chen CC, Sureshbabul M, Chen HW, Lin YS, Lee JY, Hong QS, Yang YC, Yu SL. Curcumin suppresses metastasis via Sp-1, FAK inhibition, and E-cadherin upregulation in colorectal cancer. *Evid Based Complement Alternat Med*, 2013, 2013: 541695.
- [21] Chen HW, Lee JY, Huang JY, Wang CC, Chen WJ, Su SF, Huang CW, Ho CC, Chen JJ, Tsai MF, Yu SL, Yang PC. Curcumin inhibits lung cancer cell invasion and metastasis through the tumor suppressor HLJ1. *Cancer Res*, 2008, 68(18):7428–7438.

### Corresponding author

Carmen Elena Cotrutz, Professor, MD, PhD, Department of Cell and Molecular Biology, “Grigore T. Popa” University of Medicine and Pharmacy, 16 Universităţii Street, 700115 Iassy, Romania; Phone +40730–030 302, e-mail: cotrutz@yahoo.com

*Received: April 6, 2014*

*Accepted: January 8, 2015*