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Cytotoxic assessment of Cn-AMP$_1$ in 2D or 3D spheroid non-small cell lung cancer models

Lueacha Tabtimmai[1], Anna M. Grabowska[2], Kiattawee Choowongkomon[3], Sirikhwan Tinrat[1] and Saranya Sedtananun[1,*]

[1]Department of Biotechnology, Faculty of Applied Science, King Mongkut's University of Technology North Bangkok, Bangkok, Thailand
[2]*Ex Vivo* Cancer Pharmacology Centre of Excellence, Cancer Biology, Division of Cancer and Stem Cells, School of Medicine, Biodiscovery Institute, University of Nottingham, Nottingham, United Kingdom
[3]Department of Biochemistry, Faculty of Science, Kasetsart University, Bangkok, Thailand
[*]Corresponding author: saranya.s@sci.kmutnb.ac.th

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Abstract

Three-dimensional (3D) cellular spheroids have advantages for use in drug screening because they recapitulate cell-cell interactions relevant to important aspects of tumor biology. Non-small cell lung cancer is the most common form of lung cancer. Here, A549 cells were used for 3D spheroid establishment. The number of cells and culturing time were optimized to achieve optimal, reproducible spheroid formation. Use of 1 x 10^3 cells/well elicited the highest cell viability at day 5. In the presence of non-gelling concentrations of extracellular matrix, cell viability was slightly improved compared to extracellular matrix (ECM) - deprived conditions. Immunofluorescent staining revealed proliferative cells at the rim of the spheroids (Calcein-AM stained), whereas necrotic cells gradually increased over time at the core of the spheroid in both conditions. For drug assessment, cationic coconut antimicrobial peptide (Cn-AMP$_1$) which was previously reported to be a promising peptide against colorectal cancer, was used. As anticipated, Cn-AMP$_1$ decreased the cell viability of A549 in a dose-dependent manner in the Two-dimensional (2D) cell culture model. However, in parallel experiments in 3D, A549 spheroids, Cn-AMP$_1$, did not demonstrate any cytotoxic effect. The 3D spheroid model might be feasible for use as a preclinical model for cytotoxicity assessment leading to a reduction in the use of animals in research.

Keywords: Lung cancer, Spheroid, 3D cell culture, Antimicrobial peptide, Cn-AMP1

1. Introduction

Two-dimensional (2D) cell culture is a basic model that can be used for investigation of the *in vitro* anticancer activity of natural compounds due to its simplicity, reproducibility, and cost-and time-effectiveness [1]. Unfortunately, 2D cell culture lacks the important cellular microenvironment and physiological features of real tumors [2]. Three-dimensional (3D) cell culture models have been established for various applications, and use of multicellular spheroids (MCSs) is one approach that better reflects key aspects of the *in vivo* cellular microenvironment such as complexity, heterogeneity, cell-cell/cell-matrix interactions and cellular depolarization [3]. Thus, 3D cell culture models provide an important transition between 2D cell culture model and animal models. [4]. Nevertheless, the evaluation of anticancer agents from food and agricultural resources in 3D models has not been extensively used.

Green coconut water is the clear liquid inside immature coconuts which is produced during the nuclear phase of coconut development. It is enriched with many biologically active secondary metabolites and compounds including the cationic coconut antimicrobial peptide (Cn-AMP$_1$). Cn-AMP$_1$ is isolated and purified from green coconut water, consists of 9 amino acids with a disulfide-free peptide and contains cationic and hydrophobic portions [5]. Cn-AMP$_1$ plays a role in a broad range of biological functions including antibacterial, antifungal, and immunostimulatory activity. Furthermore, Cn-AMP$_1$ elicited cytotoxicity against Cancer coli-2 Caucasian

colon adenocarcinoma (Caco-2) [6]. However, Cn-AMP$_1$ cytotoxicity was assessed under 2D cell culture conditions which may not reflect its effect in a more realistic *in vivo* cellular microenvironment.

In this study, 3D spheroids were established using the non-small cell lung cancer cell line, A549 as ECM-free 3D cell spheroids. Optimization of spheroid formation was initially performed, then cytotoxicity of Cn-AMP$_1$ was assessed in both conditions (2D *vs* 3D spheroid). While Cn-AMP$_1$ appeared to be effective in 2D models, it was ineffective in 3D models.

2. Materials and methods

2.1 Peptide

Synthetic Cn-AMP$_1$ peptide (SVAGRAQGM) was purchased from Synpeptide Co., Ltd (Shanghai, China). Purity of the peptide was >95%. Stock solutions were reconstituted in sterile distilled water and stored at -20℃.

2.2 Cell culture

Non-small cell lung cancer cell line A549 cells were used for the determination of cell cytotoxicity. Cells were grown as a monolayer in a Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 U/mL streptomycin at 37℃ in a humidified atmosphere with 5% CO_2. Cells were routinely replenished every 2 days.

2.3 Spheroid formation

A549 cells were grown as a monolayer and detached by trypsin. The cells were then stained with trypan blue solution and counted. The viable cells were seeded in various cell numbers (1-6 x 10^3 cells/well) into a 96-Ultra-Low Attachment U bottom plate. Each well was adjusted to a medium volume of 200 µL as the total volume. The plate was centrifuged at 300 x g for 10 min. Seeding day was counted as Day 0. To compare the effect of extracellular matrix, Cultrex® Basement Membrane Extract (BME) was added into the culture medium at a final, non-gelling concentration of 100µg/mL following the protocol as described above. Remarkably, BME needed to be performed on ice to prevent solidification before the experiment was set up. Cell viability and the size of the spheroid were measured daily using the Alamar blue assay. For size measurement, spheroids were photographed daily using a Nikon Eclipse Ti microscope with a 4x objective (Nikon limited, Surrey, UK). Cross-sectional area was determined using ImageJ.

2.4 Immunofluorescence staining

3-, 5-, and 7-day old A549 spheroids were selected for monitoring the cell population. Co-staining (Calcein-AM, Propidium Iodide, and Hoechst 33342) was performed. Briefly, at the indicated time, the medium in the well containing the spheroid was replaced with staining medium and incubated in a humidified atmosphere with 5% CO_2 for 3 h. The stained spheroids were imaged using fluorescence microscope with a 10x objective.

2.5 2D cell culture cytotoxicity assay

A549 cells (2 x 10^3 cells/well) were seeded in 96-well plates. After cell attachment, the medium was replaced with fresh medium containing various concentrations of Cn-AMP$_1$ (0.3-10 mM). Untreated wells and 10% Dimethyl sulfoxide (DMSO) were set as negative and positive controls, respectively. The experiment was done in triplicate. After 72 h, the treatment medium was replenished with fresh medium containing 0.5 µg/mL 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) and continually incubated for 3 h. After formazan formation, 50 µL of dimethyl sulfoxide (DMSO) was added for dissolving. The plates were measured using a multi-mode microplate reader at 570 nm measurement wavelength and 630 nm reference wavelength. the data were normalized and expressed as %Survival rate.

2.6 3D cell culture cytotoxicity assay

A549 spheroid were prepared as described above. After day 4, 100 µL of the medium was taken out. Cn-AMP$_1$ was the diluted with fresh medium as a 2x concentration (0.6-20 mM). One hundred µL of the fresh medium containing various concentrations of Cn-AMP1 were then added to each well and incubated for 96 h. Cell viability was measured using the Alamar blue assay. Briefly, Alamar blue dye was 5-fold diluted with fresh medium. 50 µL of the diluted dye were then added and incubated for 1 h. The plate was measured using a multi-mode microplate reader following the instruction's protocol.

2.7 Statistical analysis

Statistical analyses of all bioactivity assays were performed using Prism 6 (GraphPad Software, U.S.A.). Data points represent the average of three independent experiments with error bars presenting the mean ± standard error of mean (SEM).

3. Results

3.1 A549 spheroid can form itself without ECM-support

To establish A549 spheroids for evaluating the cytotoxicity of Cn-AMP$_1$, A549 cells were grown at various cell numbers under low-attachment conditions. Centrifugation was used to increase cell-cell interaction leading to aggregation at the bottom of the plate. By 24 h, some aggregation of A549s was observed. In general, cell viability and spheroid size gradually increased over time (Figure 1) although spheroids formed using 1 x 10^3 cells/well they reached the highest cell viability at Day 6 and then declined. The lowest number of the cells that are able to form spheroids was used for further investigation.

To investigate the influence of BME, spheroids were set up using 1x10^3 cells/well in the presence or absence of non-gelling concentrations of BME. Furthermore, to determine whether A549s form spheroids or cell aggregates under the conditions used, attempts were made to disrupt the A549 cell clusters by aspirating with a pipette. As anticipated, the spheroids tightly packed in the presence or absence of BME over time (Figure 2A). Even though, cell viability at Day 6 was slightly increased in the presence of BME (Figure 2B), A549 spheroids can be formed without BME, and even in the presence of BME some decline in the viability was observed at the later time points.

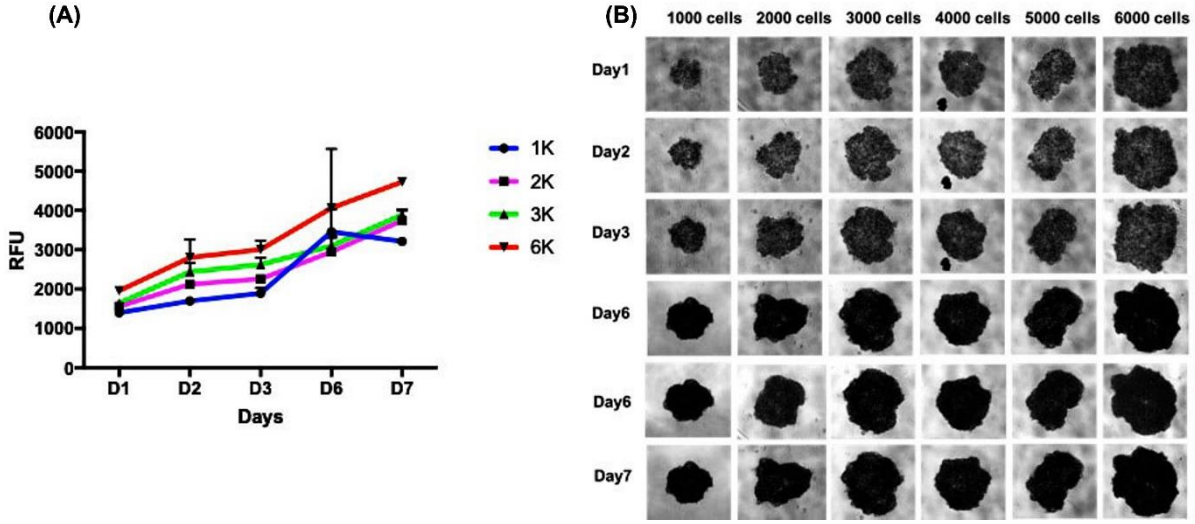

Figure 1 Optimization of A549 spheroid formation; (A) Cell viability of A549 spheroid at the indicated cell numbers were assessed by Alamar Blue assay (B) Morphological A549 spheroids were observed under an inverted microscope.

3.2 Long-term A549 spheroid culturing revealed the presence of a necrotic core

To monitor cell localization, immunofluorescent staining was performed. 1 x 10^3 cells/well, the optimal cell number, were seeded for initiating A549 spheroids. 3-, 5-, and 7- day old A549 spheroids were then selected as representative time points for monitoring. Hoechst 33342 co-staining with Calcein-AM staining revealed a proliferative cell population at the periphery (Figure 3). The Calcein-AM positive cells in the spheroids grown in the presence of BME were significantly (p = 0.0029) increased relative to cell viability results (Figure 2B) on day 5. PI-positive stained cells revealed formation of a necrotic core at the center of the spheroid in both conditions over time. Taken together, the day 6 spheroids had the highest cell viability and longer-term culturing increased the presence of the necrotic core.

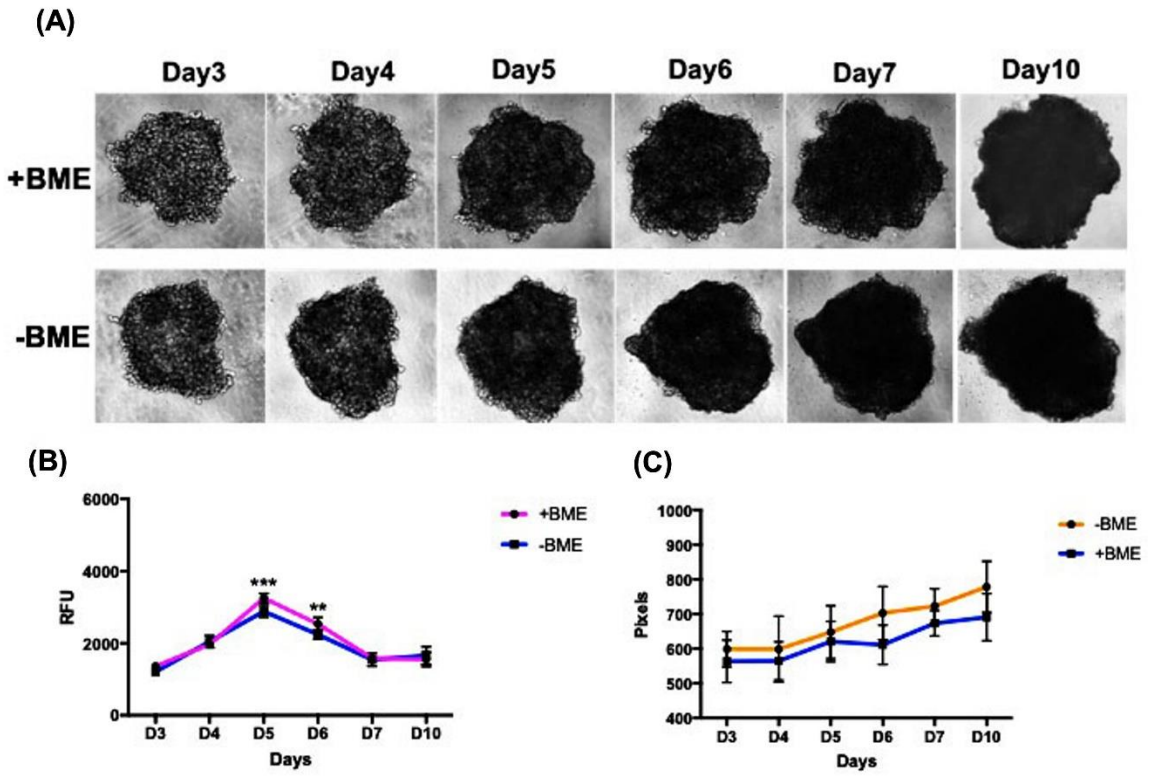

Figure 2 Evaluation of ECM-supporting effect on A549 spheroid formation; (A) Morphological A549 spheroids were observed under an inverted microscope under ECM-supporting and -deprived conditions**.** (B) cell viability and (C) size measurement was shown**.** All experiments were done in five-replicates. (** $p = 0.0021$, *$p = 0.0332$).

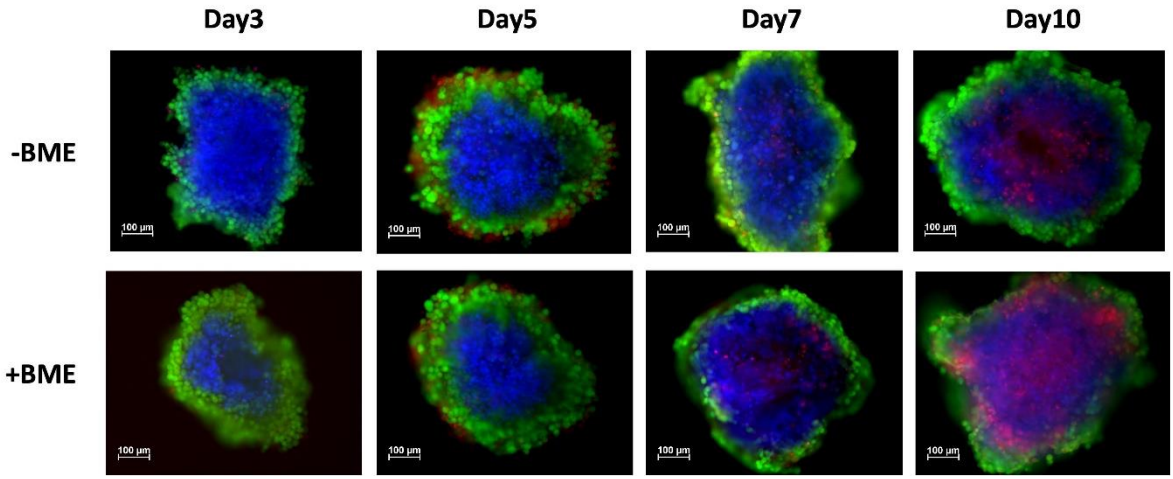

Figure 3 Cell viability was determined by immunofluorescent staining**;** cell localization (blue), proliferative cells (green), and dead cells (red) were stained under ECM-supporting and -deprived conditions at the indicated culturing day.

3.3 Cn-AMP$_1$ decreased cell viability of 2D cell culture of A549 in a dose-dependent manner

Cn-AMP$_1$ is a well-known peptide with antimicrobial activity in both gram negative and positive bacteria and also has anticancer activity. However, its mechanism is still controversial. To assess the cytotoxicity of Cn-AMP$_1$, A549 cells grown in a monolayer were treated with a range of concentrations of Cn-AMP$_1$. As shown in Figure 4, Cn-AMP$_1$ decreased cell viability of A549 at the highest dose and did not have any effect at concentrations lower than 5 mM. The inhibitory concentration value (IC_{50}) of the Cn-AMP$_1$ was estimated as 5.3±3.55 mM (Figure 5).

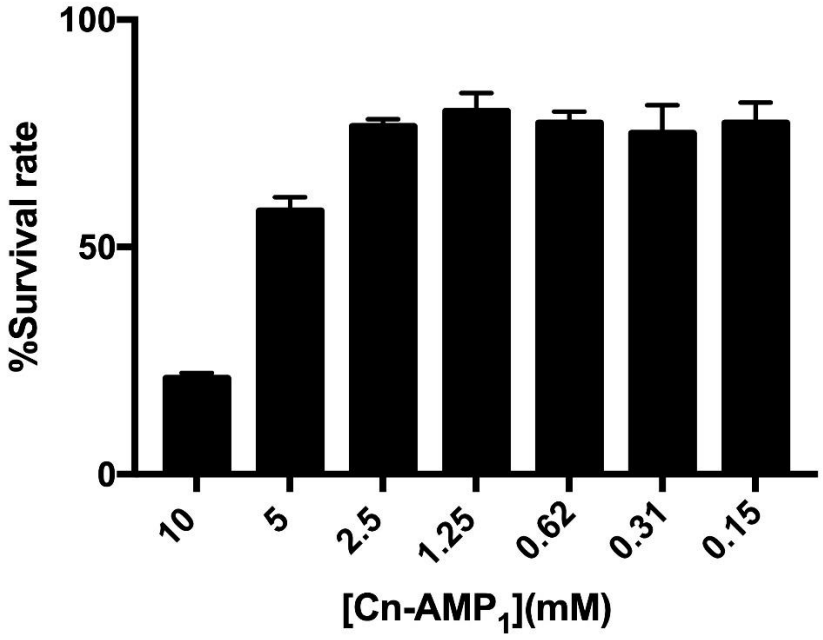

Figure 4 Cell viability of monolayer A549 after treatment with Cn-AMP$_1$ for 72 h. Data represent mean ± SEM of three independent experiments performed in five replicates.

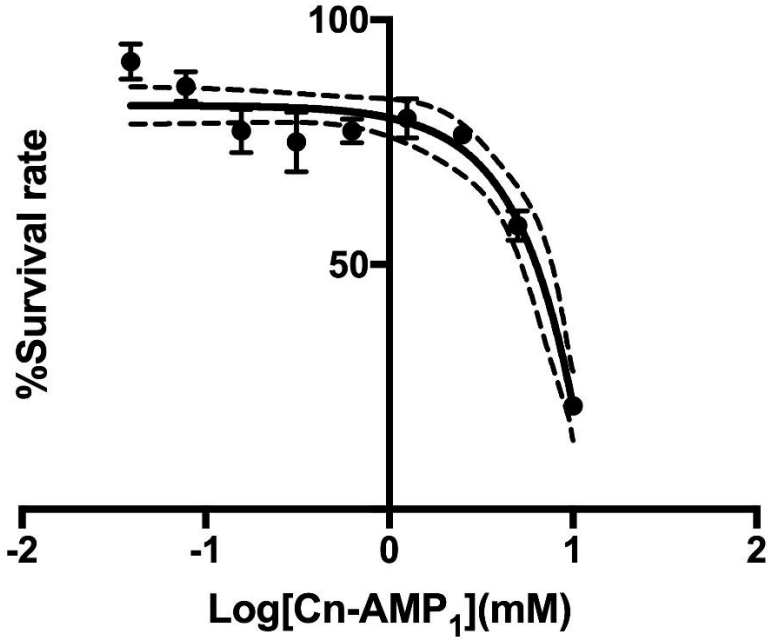

Figure 5 The inhibitory concentration value (IC_{50}) of the Cn-AMP$_1$ in 2D cell culture of A549 after treatment with Cn-AMP$_1$ for 72 h. Data represent mean ± SEM of three independent experiments performed in five replicates (R-square = 0.9245).

3.4 Cn-AMP$_1$ did not demonstrated any effects on 3D spheroid model of A549

Nowadays, the 3D spheroid model is reported to be a promising cellular model for use as a preclinical model. Cn-AMP$_1$ on the monolayer of A549 elicited the highest cytotoxicity at 10 mM. In parallel, 4-day-old spheroids were treated with various concentrations of Cn-AMP$_1$ at a comparative cell density of A549 in the monolayer. The half-inhibitory concentration (5 mM) was used to determine the effect on the 3D spheroid model. Surprisingly, Cn-AMP$_1$ did not have any effect in the 3D model (Figure 6).

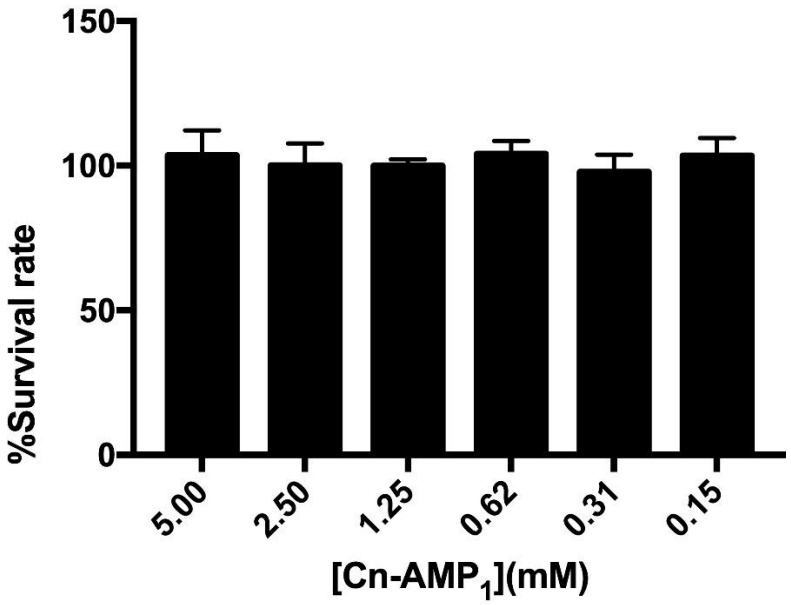

Figure 6 Cell viability of A549 spheroid model after treatment with Cn-AMP_1. Data represent mean ± SEM of three independent experiments performed in five replicates.

4. Discussion

Interest in use of cationic antimicrobial peptides in cancer treatment has increased and Cn-AMP_1, derived from green coconut milk, previously demonstrated a cytotoxic effect against Caco-2 cell line in *in vitro* 2D culture models [6]. However, 3D culture models are increasingly used for pre-clinical drug screening. Spheroids are one kind of 3D model that provides a promising platform more closely mimicking important aspects of the tumor microenvironment (TME) than conventional *in vitro* 2D models [7]. A heterogenous population of cells, nutrient/metabolism gradients, production of extracellular matrix, hypoxia and a necrotic core may be generated by culturing under low-attachment conditions [8-10]. Whilst use of such 3D culture models is still challenging for use in drug assessment [2], here, 3D A549 spheroids were established for evaluating the anticancer activity of Cn-AMP_1.

A549 spheroids have been previously used as a pre-clinical model [11-13] and a comparison of the A549 spheroid and patient-derived data revealed similar relative gene expression patterns [14]. The A549 spheroid highly upregulated several tight junction proteins that correlated with drug-resistance occurrence [15]. Extracellular matrix has been reported to support spheroid formation in some cell lines [3]. Here, we show that A549 spheroids can be formed without ECM support, although the presence of BME significantly increased cell viability at day 5/6 in 3D spheroids formed from the same number of cells.

While Cn-AMP_1 had some cytotoxic activity on the A549s in 2D culture, as previously reported for Caco-2 [6], it did not affect the 3D spheroid model. In 2D culture, cationic AMP induced cytotoxicity was involved by several mechanisms. Loss of asymmetric distribution and alteration in membrane fluidity in cancer cells increase the exposure of negative charge of phospholipid on the cell membrane [16]. Cationic peptides interact with negatively charged tumors cell membranes via electrostatic interaction. Disturbing cell membrane integrity led the death of these cells either by necrosis or apoptosis mechanisms [17]. Cationic LvHemB1 peptide was able to selectively penetrate the cancer cell membrane and target mitochondrial voltage-dependent anion channel (VDAC1) resulting in increased reactive oxygen species (ROS) and apoptosis [18]. While the cancer cells in the 3D spheroid model showed more resistance to Cn-AMP_1 than those in 2D culture. The expression of tight junction proteins in A549 spheroids could prevent peptide penetration leading to decreased cytotoxicity. Increasing Occludin-1 expression in A549 spheroids has been involved in chemoresistance [15]. It would be a reason that increasing Occludin-1 expression reduces AMP_1 diffusion into A549 spheroids leading to cytotoxicity abolishment. These findings may be relevant to the patient tumor setting where strong cell-cell interactions may also reduce drug access to tissues and, thus, lack of response [19]. Alternatively, the model should be further characterized in terms of protein expression of such drug efflux pump proteins and tight junction proteins, which may also explain the findings. Such findings will be important in determining the appropriate direction to take in further development of such drugs, including the potential need to use drug delivery systems which are better able to penetrate tissues or combination therapies to overcome drug efflux mechanisms.

5. Conclusion

Traditionally, animals have been used for screening more efficient drug or bioactive compounds in complex natural phenomena. However, the experimentation involving use of animals causes increased time, overall cost, and decreased welfare. The 3D cellular models provide a new strategy for evaluating drug efficacy; these techniques avoid the cost and legal/ethical concerns regarding the use of animals in the laboratory. More importantly, they can be used to screen drugs in more clinically relevant settings and provide valuable insight into appropriate steps that need to be taken to develop drugs towards successful clinical application.

6. Acknowledgements

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