Comparison of the apoptotic effects of bortezomib using 2D and 3D co-culture models of THP-1 derived macrophage and A549 lung cancer

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ABSTRACT: We investigated the fundamental function of the lung cancer tumour microenvironment. Proteasome inhibition has emerged as a clinically successful anti-cancer therapeutic strategy, with the antineoplastic medication bortezomib showing high efficacy against multiple cancers. Tumour-associated macrophages (TAMs), which migrate to tumour stroma, are known to promote cell proliferation, apoptosis, and metastasis within the lung cancer microenvironment. However, the specific interaction of macrophages, lung cancer cells, and bortezomib are still unclear. Most *in vitro* cell cultures are traditionally grown in a two-dimensional (2D) culture system, although 3D cultures have demonstrated greater success in terms of drug response, gene expression and viability. Therefore, to elucidate the role of macrophages, we aimed to establish a co-culture model in both 2D and 3D cultures using A549 human lung cancer cells and THP-1 derived macrophages. Apoptotic effects were analysed using the Annexin V-PI method. Since NF-kB and TNF-α play important roles in the anti-proliferation and apoptosis pathways, the levels of NF-kB and TNF-α mRNA expression were measured. As a result, bortezomib significantly increased cell apoptosis in cells co-cultured with M0 macrophages. IL-1β cytokine levels also increased, and NF-kB mRNA expression levels decreased. Understanding the mechanisms that modulate the tumour microenvironment may facilitate the development of novel anticancer therapies. The utilisation of TAMs may improve the successful treatment of lung cancer and warrants further study.

KEYWORDS: A549; macrophage; THP-1; bortezomib; 3D culture.

1. INTRODUCTION

The second leading cause of death worldwide after cardiovascular diseases, cancer is characterised as uncontrolled cell proliferation resulting from countless genetic and epigenetic anomalies [1,2]. The onset and progression of lung cancer rely on the genome development and molecular properties of cancer cells and their interaction with the tumour microenvironment [2].

The microenvironment defines non-cancer cells and tumour tissue structures, and 5–40% of the tumour mass in solid tumours consists of tumour-associated macrophages (TAMs) [3,4]. TAM cells are at the centre of the important relationship between chronic inflammation and cancer [5,6] and play a major role in cancer progression [3]. Macrophages are an essential tumour microenvironment component TAMs migrating to the tumour stream release cytokines and promote cancer cell proliferation within the lung cancer microenvironment [7]. Macrophages can express anti-tumoral (M1 macrophage) and pro-tumoral (M2 macrophage) functions in the tumor microenvironment due to their ability to differentiate into different phenotypes in response to different microenvironmental signals such as cytokines. Macrophages can be differentiated into M1 or M2 macrophages depending on the stimulus in their environment and recent studies suggest that macrophage polarization could serve as anti-cancer and anti-angiogenic therapeutic strategies [8,9] THP-1 is a monocytic human leukemia cell line that has been widely used to study monocyte/macrophage roles, signaling pathways, and drug effects. This is a distinctive feature of monocyte-macrophage-derived cells from other microenvironmental cells, which complicates their role in tumor

development [10]. THP-1 cells can be differentiated into macrophage-like cells by using phorbol-12-myristate-13-acetate (PMA), 1,25-dihydroxy vitamin D3, or macrophage colony-stimulating factor. THP-1 cells are called M0 macrophages after differentiation by PMA [11,12]. An *in vitro* co-culture model with human lung adenocarcinoma A549 cells and THP-1-derived macrophages (M0) was established to study the role of macrophages in lung cancer cell apoptosis. Because regarding apoptosis, the interaction between macrophages and lung cancer cells remains unexplained.

The ubiquitin-proteasome system is one of the most important mechanisms in protein homeostasis, and pathways involved in cell cycle regulation, growth control, and apoptosis are associated with proteasome inhibition. Bortezomib is the most studied proteasome inhibitor in drug development for non-solid malignancies. In preclinical studies, it demonstrated anti-tumour activity against solid tumour malignancies 13].

Many *in vitro* cultures have traditionally been grown in two-dimensional (2D) settings [14,15 . Cancer researchers frequently utilize 2D *in vitro* studies and small animal models to study the complex mechanisms of tumour angiogenesis, invasion and metastasis [16]. 3D models are in some ways superior to 2D models. They can be used to predict changes in cells observed during tumor progression *in vivo* and 3D cultures could contribute to cancer drug discovery due to the shortage of appropriate preclinical models [15].

This study is to adapt a 3D lung cancer model in place of the 2D model, using A549 cells within the AlgiMatrix 3D culture system. In this context to elucidate the role of macrophages in lung cancer, an *in vitro* co-culture model in both 2D and 3D culture, together with A549 human lung cancer cells and THP-1 derived macrophage was developed and the 3D culture formation optimized. Apoptotic effects were analysed by flow cytometry using the Annexin V-PI method. NF-kB and TNF-α mRNA expression levels were evaluated using real-time polymerase chain reaction (RT-PCR) due to their significant roles in anti-proliferation and apoptosis. All methods were performed and evaluated in parallel in 2D and 3D culture environments.

2. RESULTS

2.1. PMA-induced differentiation of THP-1 cells into macrophages

The effect of PMA concentrations (7.81, 15.62, 31.25, 62.5, 125, 250 and 500 ng/mL) on cell viability was investigated by the WST-1 method ((Figure 1A and Figure 1B). The highest concentrations of PMA (500 ng/mL) significantly decreased the viability of THP-1 cells at the end of 24 and especially, 48 hours of incubation, , at which time the cell viability had decreased to below 50% (Figure 1) PMA concentrations in the range of 7-250 ng/mL demonstrated no significant cytotoxic effects.Considering these data and those from previously published studies non-cytotoxic PMA concentration of 100 ng/mL was applied to THP-1 cells for 24 hours to differentiate cells into macrophages. THP-1 cells were exposed to 100 ng/mL PMA for 24 hours, and macrophage cells were photographed with a Leica DM 300 Inverted microscope. THP-1 cells exhibited a wide and round morphology and grew as single cells or partly in clusters in suspension (Figure 2A and Figure 2C). The THP-1 cells were differentiated into macrophages after stimulation with 100 ng/mL PMA for 24 h. During this differentiation process, the cells were attached to the bottom of the flask. At the same time, their morphology changed from round to almost spindle-shaped, and in some cells, pseudopods were observed (Figure 2B and Figure 2D).
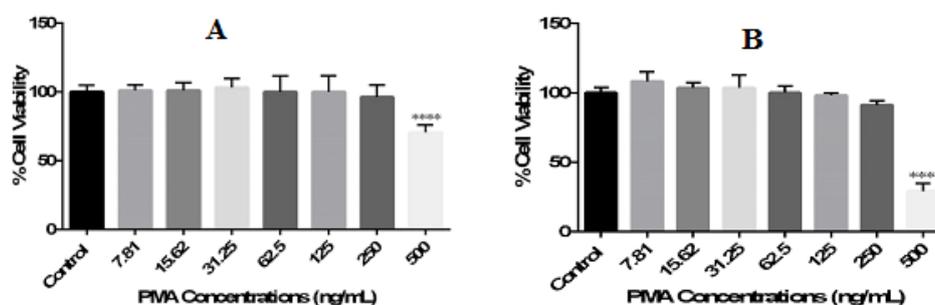

Figure 1. Cytotoxic effects of PMA on THP-1 cells. The cells were treated with various PMA concentrations for 24 (A) and 48 (B) hours, and cell cytotoxicity was determined by WST-1 assay (Control: 0.1% DMSO containing medium, ±: S.D., p<0.0001****). Experiments were performed in triplicate independently and used 8 wells for each group.
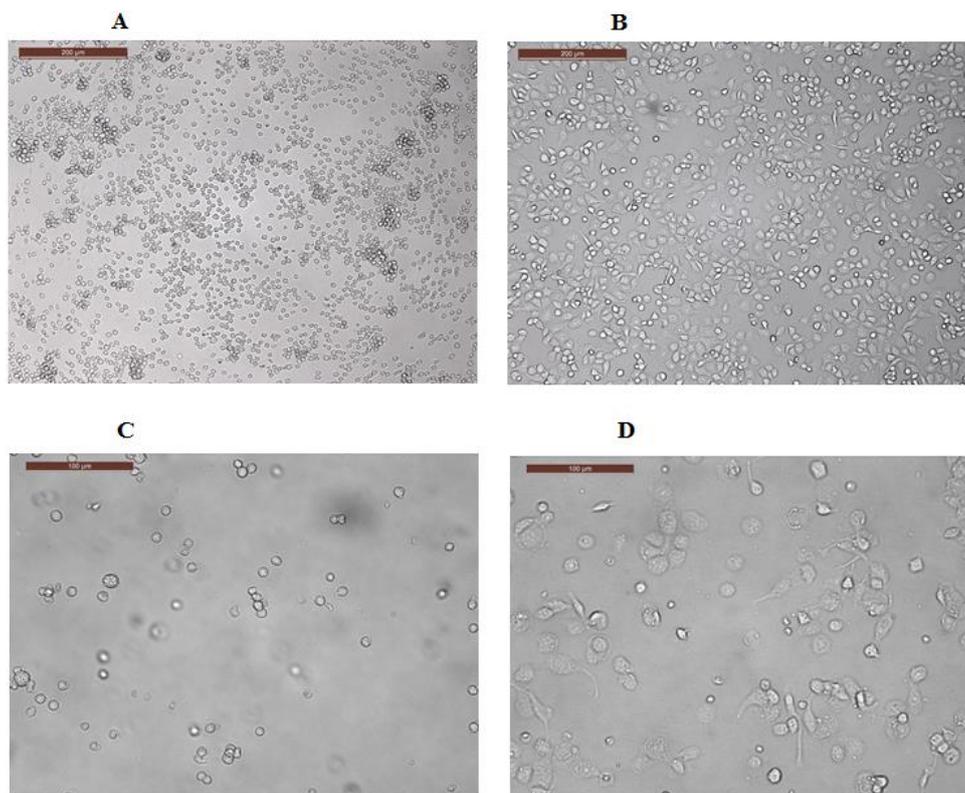
Figure 2. Microscope image of THP-1 cells (A: 10X objective, C: 20X objective) and THP-1 derived macrophages (B: 10X objective, D: 20X objective).

2.2. Evaluation of bortezomib cytotoxicity on THP-1 derived macrophages

THP-1 cells were seeded at a density of 1×10^4 into 96-well plates with 100 ng/mL PMA in the medium. After THP-1 cells differentiated into macrophages (by 24 hours), the cells were incubated for 48 hours with the standard medium. Bortezomib (1.56, 3.125, 6.25, 12.5, 25, 50, 100 µM) was then applied for 48 hours to determine its effect on cell viability. No applied concentrations of bortezomib decreased the viability of M0 macrophage cells below 50% (**Figure 3**). These results demonstrate that the bortezomib concentration to be used in the co-culture study (concentrations of 100 µM and below) did not have a cytotoxic effect on M0 macrophages, and thus M0 macrophages contributed to the production of cytokines secreted by the macrophages in the co-culture process.
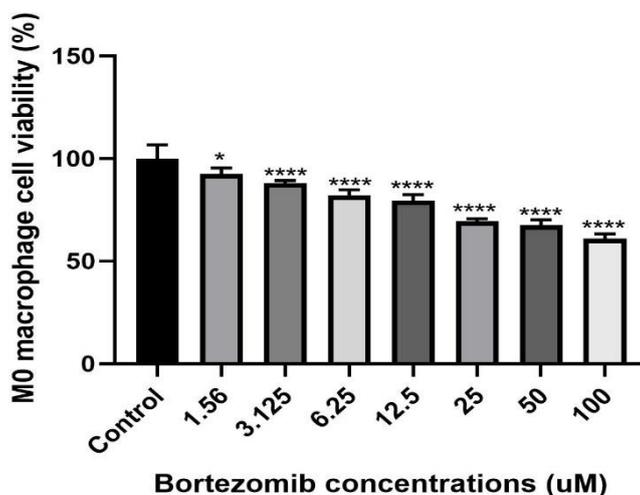

Figure 3. Cytotoxic effects of bortezomib on M0 macrophages. The WST-1 assay was used to determine cell cytotoxicity after 48 hours of treatment with different concentrations of bortezomib (n=8, ±: S.D., Control: 0.1% DMSO containing medium, $p < 0.05^*$, $p < 0.0001^{****}$).

2.3. Evaluation of 3D cell cultures

In our study, a 3D cell culture model was created using A549 cells and the special 96- and 6-well plates of the AlgiMatrix® 3D Culture System. A549 cells are ready for 3D culture experiments 7 days after cell seeding. The 7-day 3D culture spheroids are shown in **Figure 4A**. On the 7th day, the spheroids of cells in randomly selected wells were stained with Hoechst 33258, and fluorescence imaging (**Figure 4B**) was performed using a Cytation 3 Cell Imaging Multi-Mode Reader.
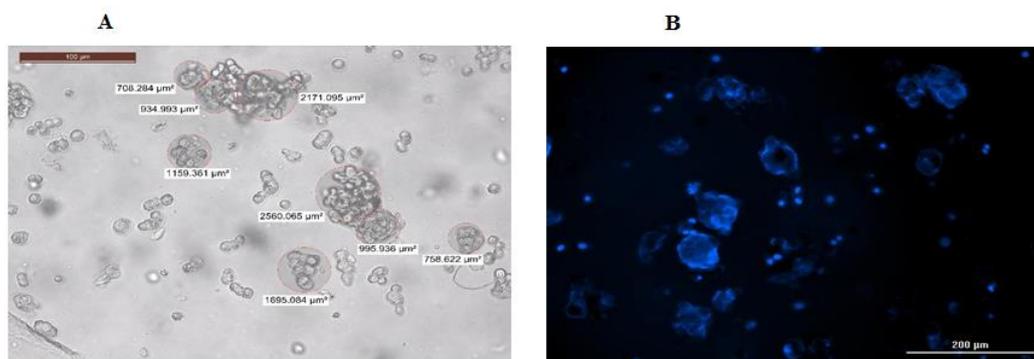

Figure 4. Microscope image of 3D cell culture. Spheroid analysis (A) and Hoechst 33258 imaging (B) of A549 cells on day 7 after seeding on Algimatrix plates (10X objective).

2.4. Flow cytometry evaluation of apoptotic effects using the annexin V-PI method

After establishing the 2D and 3D co-culture models of A549 cells and M0 macrophages, A549 cells were exposed to the IC_{50} concentrations of bortezomib (6.32 μM and 98.6 μM for 2D and 3D cultures, respectively) determined in previous studies for 48 h. After applying the Annexin-V Fitc Apoptosis Detection Kit (Catalog no: 556547, BD) protocol on cells in each of the 6 wells, the samples were analysed in a flow cytometry device (Accuri C6, BD). The apoptotic (early apoptosis + late apoptosis) effect of bortezomib increased in both 2D and 3D cultures, compared to A549 and M0 macrophage co-culture control groups (**Figure 5** and **Table 1**). The apoptotic effect of bortezomib was 41% in the 2D culture co-culture group (36.6 +4.4) and 50.3% (20.1 + 30.2) in the 3D co-culture. While the effect of bortezomib on cell necrosis in 2D culture in the A549 co-culture group was 21.5%, this rate decreased to 2.9% in 3D culture. This 10-fold difference showed that bortezomib did not cause 3D culture necrosis of A549 cells.

Table 1. Apoptotic effect percentages of bortezomib in A549 cells determined by flow cytometry and Annexin/PI method.

	Groups	Viable cells %	Necrotic cells %	Early apoptotic cells %	Late apoptotic cells %
2D	A549 Control	91±0.8	6.8±1.3	1±0.5	1.2±0.8
	A549 Bortezomib	74.5±4	5.4±1	1.9±0.5	18.2±3.6
	M0 Macrophage+A549 Control	89.7±0.6	1.1±0.1	5.7±0.4	3.6±0.4
	M0 Macrophage+A549 Bortezomib	37.4±2.7	21.5±2.1	4.4±3.7	36.6±2.2
3D	A549 Control	90.9±0.7	1.2±0.4	7.3±1.5	0.6±0.3
	A549 Bortezomib	69.9±2.3	1.8±0.3	6.4±2.1	21.9±1.1
	M0 Macrophage+A549 Control	90.6±0.3	1±0.1	2.7±0.9	5.8±0.6
	M0 Macrophage+A549 Bortezomib	46.8±2.1	2.9±3.6	20.1±1.4	30.2±2.5

2.5. Flow cytometry evaluation of cytokine levels

Levels of cytokines IL-12p70, TNF-α, IL-10, IL-6, IL-1β and IL-8, as determined by flow cytometry in A549 lung cancer cells, were not significantly different between the 2D and 3D culture media. As expected, TNF-α levels increased in M0 macrophages and A549 co-culture groups in both 2D and 3D culture compared to the A549 control group. The highest levels of IL-6 in 2D and 3D cultures were 1206.16 pg/mL and 1129.53 pg/mL in M0 macrophages and A549 co-culture groups, respectively. Additionally, bortezomib caused an approximately 20-fold increase in IL-1β levels in both 2D and 3D cultures. The highest IL-8 levels were determined in 2D and 3D cultures of M0 macrophage and A549 co-culture groups. Bortezomib reduced the IL-8 level by 1.5-fold in both 2D and 3D cultures in the co-culture groups (**Table 2**).
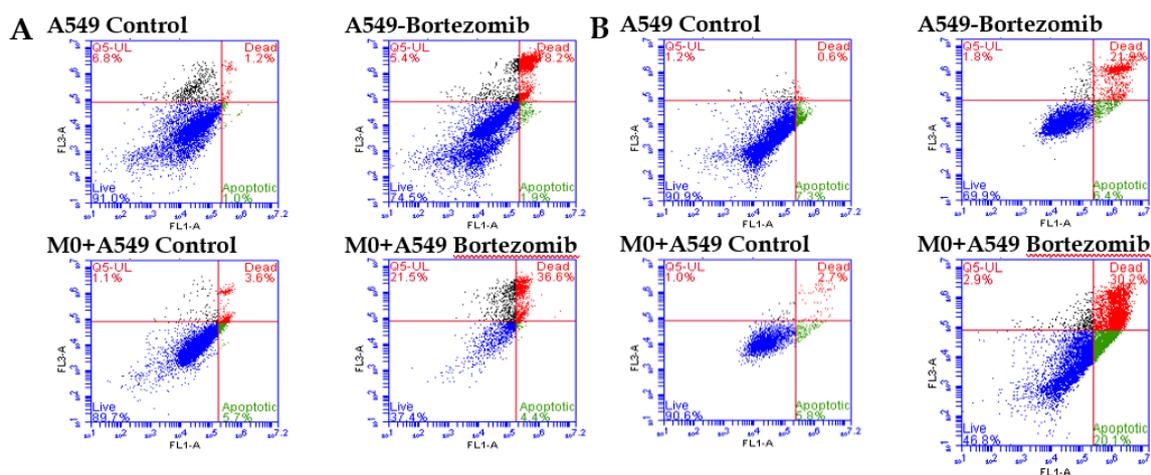
Figure 5. Determination of the apoptotic effect of bortezomib by flow cytometry. Single and co-culture groups in A549 cells in 2D (A) or 3D (B) cultured for 48 hours with bortezomib concentration. At least 10,000 cells were analyzed per sample and quadrant analysis was performed. The proportion of cell number is shown in each quadrant viable cells, necrotic cells, early apoptotic cells and late apoptotic cells (%). Experiments were performed in triplicate independently.

Table 2. Cytokine levels of bortezomib in A549 cells determined by flow cytometry and cytometric bead array technology (pg/mL).

	Groups	IL-12	TNF	IL-10	IL-6	IL-1Beta	IL-8
2D	**A549 Control**	1.23	0	1.57	54.72	0	2739.74
	A549 Bortezomib	0.41	0	0	349.09	0	3769.36
	M0 Macrophage+A549 Control	0	12	3.05	1206.16	77.57	34105.84
	M0 Macrophage+A549 Bortezomib	0.44	11.36	0.52	1111.72	1571.55	20671.35
3D	**A549 Control**	0	0.41	0	132.49	-	8032.47
	A549 Bortezomib	0.35	0	0	281.68	1.34	6880.01
	M0 Macrophage+A549 Control	0.44	12.64	1.67	1129.53	60.86	30949.69
	M0 Macrophage+A549 Bortezomib	0.44	11.68	0	1024.43	1507.83	19402.96

2.6. Evaluation of NF-kB, TNF-α, mRNA expression levels by RT-PCR method

mRNA levels of NF-kB and TNF-α genes in A549 cells were determined by normalising them to the control group by RT-PCR. Bortezomib in 2D culture resulted in 4.13 and 5.27-fold (single culture) and 8.19 and 4.82-fold (macrophage co-culture) decreases in NFKB and TNF-α mRNA gene expression levels, respectively, compared to the control. When the effects of bortezomib on A549 cells were compared between the groups in 3D culture, it was determined that NF-kB and TNF-α gene expression decreased 3.39 and 1.03-fold in the single culture group and 104.1 and 312.5-fold in the co-culture group, respectively (**Table 3**).

3. DISCUSSION

The apoptotic effects of the proteasome inhibitor bortezomib were investigated in this study using a co-culture model of THP-1 derived macrophage cells and A549 lung cancer cells. The anti-apoptotic effects of bortezomib on A549 lung cancer cells were also compared using 2D and 3D culture methods.

THP-1 is a human leukemia monocytic cell line widely used to study monocyte/ macrophage functions, mechanisms, signaling pathways, and the pharmacological action of drugs. Studies have reported that THP-1 human monocyte cells are differentiated into macrophage cells using various stimulating agents such as PMA [10,11,12]. Consequently, the PMA concentration (100 ng/mL) and incubation time we determined in our study are in parallel with many studies [11,17].

Table 3. Normalised values and fold changes for NF-kB and TNF-α genes according to the A549 control group.

	Groups	NFKB	Fold Change (NFKB)	TNF-α	Fold Change (TNF-α)
2D	**A549 Control**	**1.000**		**1.000**	
	A549 Bortezomib	0.2420	⬇ 4.13	0.1221	⬇ 8.19
	M0 Macrophage+A549 Control	5.785	⬆ 5.78	16.57	⬆ 16.57
	M0 Macrophage+A549 Bortezomib	0.1896	⬇ 5.27	0.2074	⬇ 4.82
3D	**A549 Control**	**1.000**		**1.000**	
	A549 Bortezomib	0.2944	⬇ 3.39	0.9645	⬇ 1.36
	M0 Macrophage+A549 Control	0.1032	⬇ 9.68	0.0222	⬇ 45.04
	M0 Macrophage+A549 Bortezomib	0.0096	⬇ 104.16	0.0032	⬇ 312.5

Proteasome inhibitors inhibit proteasome activity, restrict protein synthesis, and downregulate the expression of target gene such as NFkB, thereby suppressing cancer cell proliferation [12]. Bortezomib, is a reversible boronic acid that blocks chymotrypsin-like activity in the proteosome. The FDA approved bortezomib (Velcade, PS-341) for the treatment of multiple myeloma [13,14]. Bortezomib has been shown to have anti-tumor potential in solid tumors in studies [18,19]. Apoptosis is a controlled and programmed cell death that is activated by intracellular and extracellular pathways and regulated by many proteins [20]. Proteasome inhibitors have been reported in previous studies to cause apoptosis in cancer cells [21,22,23]. In addition, many studies have reported that interactions with tumor-associated macrophages may alter the proliferation of cancer cells and their response to chemotherapy agents [9]. According to our Annexin V-PI results, IC_{50} concentrations of bortezomib have been shown to increase A549 cancer cell apoptosis in the presence of M0 macrophages in both 2D and 3D culture.

Previous research has shown that tumor-associated macrophages manufacture cytokines such as IL-1β, IL-8, and IL-6. [24, 25]. In our study, the high levels of the same cytokines obtained from the A549 cancer cell and M0 macrophage co-culture support other studies. In previous studies, there was no evidence investigating the effect of bortezomib on IL-1β levels in A549 cells. However, in another study, it was reported that bortezomib, a pharmacological inhibitor of NF-κB, increased IL-1β protein levels in lung cancer cells isolated from mice, in line with our results [26]. Also, in another study conducted in A549 lung cancer cells, it was reported that bortezomib suppressed NF-kB and IL-8 at the protein level [27]. This showed the effects of bortezomib on both apoptosis pathways and IL-8 when evaluated, and it has shed light on the M0 macrophage and A549 co-culture model.

In our study, "AlgiMatrix® 3D Culture System" was used to create a 3D culture environment. The porous nature of the AlgiMatrix® system provides the necessary space for cells to grow as models of *in vitro* lung tumors and other organ types based on the type of cells incorporated [14]. Similar to this study, we optimized our study in terms of cell number and incubation times (data note shown). In our study, the antiapoptotic effects of bortezomib in A549 lung cancer cells in 2D and 3D environments were determined for the first time and compared. As the 3D culture environment is closer to *in vivo* conditions, these concentrations will shed light on future studies. In the light of the results we obtained from this study, M0 macrophage polarization was realized from THP-1 monocyte cells, and we obtained an optimized differentiation model for our next M1 and M2 macrophage polarization studies and co-culture models. As a result, it is the first research to compare Bortezomib's apoptotic effects in 2D and 3D lung cancer cells in single and macrophage co-culture models.

4. CONCLUSION

Our findings of bortezomib's strong apoptotic effects on A549 cells in the presence of THP-1-derived M0 macrophages could inform the use of TAMS and bortezomib in the treatment of lung cancer. However, it is essential to demonstrate the effects of bortezomib on additional cell lines, other members of tumour-associated macrophages such as M1 and M2, and mouse models of human lung cancer. As a result, elucidation of additional molecular pathways associated with apoptosis and proteasome inhibition is required to evaluate bortezomib treatment of lung cancer.

5. MATERIALS AND METHODS

5.1. Cell culture and treatment

A549 and THP-1 cells were provided from American Type Culture Collection (ATCC) (A549: CCL-185™ and THP-1: TIB-202) and were grown in RPMI 1640 medium containing L-glutamine and 10% foetal bovine serum, 1% penicillin/streptomycin at 37 °C in a humidified incubator with a 5% CO_2 atmosphere. Bortezomib was dissolved as a stock solution in dimethyl sulfoxide (DMSO).

5.2. Differentiation of THP-1 macrophages

A 4-[3-(4-Iodo-phenyl)-2-(4-nitrophenyl)-2H-5 tetrazolium]-1,3-benzene disulphonate (WST-1) assay (Roche, Germany) was used to evaluate cell cytotoxicity. The WST-1 method, in which mitochondrial dehydrogenases in viable cells cleave the tetrazolium salt WST-1 in formazan, was performed on THP-1 cells to determine non-cytotoxic concentrations of PMA. THP-1 cells were seeded at a density of 1×10^4 cells/100 mL medium in 96-well culture plates. They were subsequently incubated in 100 mL of medium containing different concentrations of PMA (500, 250, 125, 62.5, 31.25, 15.62, 7.81 ng/μL) for 24 and 48 h. WST-1 (10 mL/well) cell proliferation reagent was then added to the wells, and colourimetric analysis was performed after 3 hours using a Cytation 3-Cell Imaging Multi-Mode Reader at 420 nm (Bio-Tek, Germany). Cell survival rates were calculated as a percentage of the control group. THP-1 cells and macrophage cells treated with 100 ng/mL PMA concentration for 24 hours were photographed with Leica DM 300 Inverted microscope.

5.3. Determination of the cytotoxicity of bortezomib on THP-1 derived macrophage cells

THP-1 cells were seeded at a density of 1×10^4 cells/100 mL serum-free medium/well in 96-well plates. They were subsequently induced to differentiate into a macrophage-like phenotype in 100 mL of serum-free medium containing 100 ng/mL PMA for 24 hours. After three washes, macrophages were incubated for an additional 48 hours to eliminate the effects of PMA (rest incubation) in a standard medium containing FBS. After resting, bortezomib concentrations (100, 10, 1, 0.1, 0.01, 0.001 μM) were applied for 24 and 48 hours and their effect on cell viability was determined. WST-1 cell proliferation reagent (10 mL/well) was added, and colourimetric analysis was performed after 3 hours using Cytation 3-Cell Imaging Multi-Mode Reader at 420 nm. Cell survival rates were calculated as a percentage of the DMSO (0.1%) solvent control.

5.4. 3D cell culture model

The AlgiMatrix® 3D Culture System was used to culture A549 cells in 3D. The AlgiMatrix® 3D Culture System facilitates three-dimensional (3D) cell culture and is a bioscaffold not of animal origin. Each bioscaffold is an alginate sponge with a pore size of ~50–200 μm. This cell sponge is a closer match to normal cell morphology and behaviour, an ideal solution for 3D cell culture models in cancer research.

For 3D culture application in 96- or 6-well plates, 2×10^4 or 5×10^5 A549 cells per well were incorporated into the 3D alginate scaffold in 100 or 2000 μL media, respectively. The plate was centrifuged at 100 x g for 4 minutes to ensure that the sponges were thoroughly inoculated with the cells. An additional 200 or 3000 mL of media was added after 20 minutes, and cells were cultivated at 37 °C and 5% CO_2 in a humidified incubator. Fresh media was exchanged every 48 hours. 3D culture experiments with A549 cells can be started 7 days after cell seeding.

5.5. Co-culture procedures

A549 cells and M0 macrophages were co-cultured within a cell culture insert with a 0.4-μm porous membrane (Corning, NY, USA) that separates the upper and lower chambers. Into the transwell insert's upper chamber, 25×10^5 cells THP-1 monocyte cells were seeded and treated with 100 ng/mL PMA for 24 hours to stimulate their differentiation into macrophages. Cells were washed with phosphate-buffered saline and incubated for another 48 hours in a growth medium to eliminate the effects of PMA. A549 cells were seeded at a density of 5×10^5 cells per well in the lower chamber and incubated for 7 days for 3D culture spheroid formation. When both cells were ready for the co-culture process, the M0 macrophage seeded chambers were placed onto the six-well plates containing the A549 cell spheroids and treated with a serum-free medium containing an IC_{50} concentration of bortezomib for 48 hours. A549 cells and M0 macrophages were incubated only in serum-free medium for 48 hours as the control group. Subsequently, cytokine levels from cell supernatants were determined by flow cytometry. Early and late apoptotic effects were determined by the Annexin V-PI method in flow cytometry of A549 cells, RNA was isolated, and the mRNA expression levels of the NF-kB and TNF- α genes were investigated using RT-PCR.

5.6. Apoptosis detection using annexin V-FITC and propidium iodide staining (PI)

The Annexin V-FITC Apoptosis Detection Kit (BD Pharmingen, cat. no. 556547) was used according to the kit protocol to evaluate apoptotic effects. After conducting the co-culture model as described in section 5.5 in both 2D and 3D *in vitro* environments and treating the cells with IC_{50} concentrations of bortezomib for 48 h, cells were resuspended with 100 mL PBS and stained with 5 mL Annexin V-FITC solution and 5 mL propidium iodide (PI) solution for 20 min at room temperature. At the end of the incubation, samples were diluted with 250 ml of the 1X binding buffer, processed and analysed on the Accuri-C6 (BD) flow cytometer for data acquisition; at least 1×10^4 cells were analysed per sample [12].

5.7. Cytokine detection by BD cytometric bead array technology

The BD™ Cytometric Bead Array (CBA) Human Inflammatory Cytokines Kit was used to detect cytokines, thereby investigating the anti/pro-inflammatory effects of bortezomib. After the co-culture process, cytokine levels from cell supernatants were determined by flow cytometry. The 2D and 3D single cultures were incubated with IC_{50} concentrations of bortezomib, and the co-culture system and cell supernatants were collected and stored at -20 °C. The capture of interleukin-8 (IL-8), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10), tumour necrosis factor (TNF) and interleukin-12p70 (IL12p70) proteins was performed in six bead populations with different fluorescence intensities. All six groups of beads were mixed to form the CBA, resolved in a red channel of the flow cytometer (FL3 or FL4). IL-8, IL-1β, IL-6, IL-10, TNF and IL12p70 protein levels were measured using FCAP Array v 3.0 multiplex analysis software [24].

5.8. Determination mRNA expression levels by RT-PCR

Total RNA was isolated from A549 cells using the MagNA Pure Compact RNA Isolation Kit (Roche, Reference no: 04 802 993 001) following the 2D and 3D co-culture models to investigate the mRNA expression levels of NF-κB and TNF-α genes of the samples with RT-PCR. The MagNA Pure LC 2.0 system RNA was used for isolation according to the manufacturer's protocol. A total of 500 ng RNA was used for cDNA synthesis for each RNA population using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Reference no: 05 081 963 001) process. The total cDNA was then used in RT-PCR to determine the NF-κB and TNF-α mRNA expression levels. GAPDH mRNA expression levels served as an internal positive control. Primer sequences of NF-κB1 and TNF-α were 5'-CGCCGCTTAGGAGGGAGA-3', 3'-GGTATGGGCCATCTGCTGTT-5' and 5'- GCCCTACTATTCAGTGGCGA-3', 3'- GAGCTTCTTCCCACCCACAA-5', respectively. Expression levels were determined using the SYBR® Green compatible primers and reagents (Sybr Green Master Mix, Roche) and performed in a Light Cycler 480 (Roche, Germany). Samples were tested in triplicate analyses, and specific mRNA levels were quantified and compared to GAPDH (5'-GTCAAGGCTGAGAACGGGAA-3', 3'-AAATGAGCCCCAGCCTTCTC-5') using analysis software (version 1.5.0) designed for the instrument [8,12].

5.9. Statistical analysis

GraphPad Prism 6.0 program was used to draw statistical evaluations and graphs. Data were analysed by one way ANOVA with Tukey's post-hoc and expressed as mean ± standard error, and p-values <0.05*, <0.01**, <0.001*** and <0.0001**** were considered significant.

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