Research Article

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Journal of Clinical Medicine and Therapeutics

2017
Vol. 2 No. 3: 19

The Effect of cRGDfV on Behaviour in 3D Spheroid Invasion Assay and 2D Models

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Abstract

Tumour dissemination is a major reason for failure of therapy for many tumour types therefore there is a requirement for novel targets & therapies. One of the main challenges is having a relevant, validated experimental model that expresses the tumour dissemination process. The aim of the work presented here is to study the effect of the compound cRGDfV on the invasion of U87-MG cell as spheres in 3D model and as single layer in 2D model. cRGDfV inhibited U87-MG spheroid invasion through the gel in a dose and time-dependent manner. However, cRGDfV showed a significantly more potent effect on the invasion of U87-MG cells through Matrigel coated 2D transwells compared to its effect on the invasion of U87-MG cells from spheroids in collagen. The spheroid invasion assay has the advantage over standard monolayer transwell chamber invasion assays of being a 3-dimensional assay, and thus mimics better the cell-cell interactions and architecture that are present in a tumour compared to the monolayer-based assay.

Keywords: Cancer; Invasion; Migration; Spheroids; Transwells; Pharmacology; Metastasis

Received: July 03, 2017; **Accepted:** July 28, 2017; **Published:** August 04, 2017

Citation: Zraikat M, Shnyder S, Sheldrake H, Patterson L (2017) The Effect of cRGDfV on Behaviour in 3D Spheroid Invasion Assay and 2D Models. J Clin Med Ther. Vol. 2 No. 3:19

Introduction

Cancer is a major human health problem worldwide and cancer dissemination is the major cause of death for most kinds of cancer [1].

Metastasis and angiogenesis result from the interaction between the tumour cells in the tumour microenvironment. The detailed picture of tumour and the tumour microenvironment interaction however is not fully understood due to a lack of representative models [2].

Whilst the current cancer therapies have demonstrated considerable success, there are still many fatalities from the disease. This can be explained by several reasons including inability to prevent or control tumour dissemination once it happens, the development of drug resistance, and the off-target toxic effects on normal cells [3].

The tumour dissemination process covers metastasis, including migration, invasion, adhesion, and angiogenesis. Metastasis is a multistep process which involves the detachment of cells from the primary tumour, movement of the cells on the basement membrane (migration), and penetration through the basement membrane by degradation of the ECM proteins (invasion) [4].

Invasion is a critical feature of malignancy which involves alteration of many proteins [5]. There is a large range of invasion models, all with different strengths and weaknesses. The model which we will focus on in this work is the *in vitro* 3D spheroid invasion in collagen. The 3D assay was originally developed by Ref. [6] and since been used extensively by many authors [7-10]. The advantage of this assay is that it gives an indication of chemo-resistance and other therapeutic problems that are associated with the delivery of the compounds due to gradients in proliferation, nutrients and oxygen supply within the spheroids which mimics the situation seen in the tumour mass. Furthermore, this assay gives a clear idea about cell-ECM interactions which mimic cell-ECM interactions *in vivo* [9].

The 3D invasion model will be compared with the 2D monolayer transwell chamber invasion assays through testing the effect of the same compound cRGDfV on the invasion of U87-MG spheres in the 3D model and on the invasion of U87-MG monolayer in the 2D model.

The aim of this work is to give an idea about the importance of 3D invasion assay in giving more realistic testing of the ability of the

compounds to suppress tumor invasion. Furthermore this work aims to give an idea about the importance of the collaboration between the 2D and 3D assays in understanding the functions and problems of the ant invasive compounds.

Material and Methods

Materials

The compound: The integrin inhibitor cRGDfV was obtained from Enzo Life Science (Farmingdale, Norway). For the MTT cytotoxicity assay, cRGDfV was prepared at a range of concentrations from1 nM-100 uM as final dilutions using RPMI 1640 cell culture medium as a diluent. The MTT solution was prepared by diluting the MTT stock solution (5 mg/ml) ten times using (RPMI) 1640 full media.

The cell line: The U87-MG Glioma cell line was obtained from the European Collection of Cell Cultures ((ECACC), Salisbury, Wiltshire, England). Cells were maintained in RPMI 1640 medium, supplemented with 10% foetal bovine serum, 2 mM Lglutamine, and 1 mM sodium pyruvate to form complete growth medium. Cell lines were maintained at 37°C in a 5% CO_2 humidified atmosphere.

Regents and solvents: All the reagents used in MTT assay were purchased with high purity from Sigma-Aldrich (Poole, UK). Collagen I was purchased from Sigma, ultra-pure bovine, catalogue number C4243. Matrigel Matrix was purchased with high purity from Corning Cat. No. 354234.

Methods

MTT cytotoxicity assay: All the materials used in this assay were purchased from Sigma-Aldrich (Poole, UK). The U87-MG cells were seeded in log phase at low passage number [2-5]. The MTT Cytotoxicity assay was performed in triplicate wells per treatment concentration. The U87-MG cells were seeded in a 96-well plate (1 x 10^3 cell/180 µL/well) and incubated overnight at 37°C, 5% CO_2. Serial dilutions were prepared and added to each well in at 10X on-plate concentration and incubated at 37°C, 5% CO_2 for 4 days. The MTT solution was prepared by adding 2 ml of MTT stock solution (5 mg/ml) to 18 ml of complete RPMI 1640 media in a 30 ml tube. The supernatant was removed and 200 µL of the MTT solution per well was added and incubated at 37°C, 5% CO_2 for 4 hrs. The MTT solution was removed and 150 µL of DMSO were added to each well to dissolve the formed blue formazan crystals. The optical density of the plates was read at 550 nm. The optical density was used to calculate the% survival of the compound according to the following equation:

$$Percent\ survival = \frac{Average\ of\ the\ optical\ density\ of\ the\ wells\ treated\ by\ the\ compound - blank}{Average\ of\ the\ optical\ density\ of\ control\ (no\ treatment) - blank} \times 100$$

The IC_{50} which is the compound concentration that causes a decrease of the% survival to 50% was calculated after plotting the compound concentration versus the**%** survival.

Collagen invasion assay: According to Ref. [7]; enough collagen to allow for 1400 µL per 8 wells was made as follows; 1400 µL Collagen I was added to a 1 ml Eppendorf tube on ice. Collagen I was supplemented with 0.2 ml of complete RPMI. Then sufficient amount of 1 N NaOH was added to Collagen I to reach a pH 7.4. The pH was measured by pH-indicator paper, 6.4–8.0 (VWR International, JENCONS, UK). The collagen mixture was pipetted into each chamber of an 8-chamber cover glass (Nunc, Lab-TeK, Thermo scientific). After polymerization at 37°C for 45 min, spheroids from hanging drops were carefully removed using a 20 µL pipette set at 5 µL volume and 1 spheroid was placed in each chamber of the cover glass. The medium was then allowed to evaporate in the cabinet so that the spheroid could adhere to the collagen. Another 200 µL layer of collagen gel matrix was prepared as above and carefully added to each chamber and then set in a 37°C incubator (no CO_2) for 45 min to polymerize. Finally 200 µL of RPMI was added to each chamber and incubated at 37°C, 5% CO_2 for 7 days. The spheroids were observed using inverted light microscopy at 10X objective lens and images were captured daily using Nikon Eclipse camera (TE2000-4, Panasonic Lumix DMC-GF6).

Data analysis: The analysis of the collagen invasion assay was performed by evaluating the spheroid invasion area using ImageJ. The t-test was used in addition to the Kruskal-Wallis test and Conover-Inman test to do the statistical analysing of the data, with statistically significant results of p values < 0.05 and p < 0.01.

The 2D invasion model: The 2D transwell invasion assay was performed as a way to compare the results of this assay with the results obtained from the 3D spheroid invasion assay.

Coating buffer and matrigel preparation: Coating buffer of 0.01 M Tris (pH 8.0) and 0.7% NaCl was prepared and filtered using a 0.2 µm sterile filter unit, allowed to freeze at -20°C and used after 2 hrs of freezing. Matrigel Matrix (Corning Cat. No. 354234) aliquot was thawed on ice at 4°C. All pipettes, syringes, and containers that came in contact with Matrigel Matrix were chilled prior to use. Matrigel was mixed with the prepared coating buffer to a final concentration of 250 µg/ml and placed on ice.

2D transwell assay preparation: The lid from a 24-well permeable support plate (Corning Cat. No. 3422) was removed and using sterile forceps the required number of permeable supports was transferred into a 24-well plate. 0.1 ml of the diluted Corning Matrigel Matrix coating solution was added to each permeable support with avoidance of air bubble formation while pipetting. The coated invasion chambers were incubated at 37°C for 2 hrs [11]. A U87-MG cell suspension of 5 x 10^4 cells/mL in culture medium was prepared and seeded in 150 µL volume into the upper section of the 24 well invasion chambers. (0.75 mL) of HBSS or 1% FCS or 10% FCS were added as chemo attractant to the wells of the 24-well plate via the access port. The cell invasion chambers were incubated overnight in a humidified tissue culture incubator at 37°C, 5% CO_2 atmosphere.

Transwell processing: After completing the incubation time, a cotton swab moistened with medium was inserted into the top of the Corning® Matrigel® Matrix coated permeable support (apical side) and applied gently with firm pressure to rub the area. The cells on the lower surface of the membrane were fixed by 70% ethanol for 2 min and allowed to dry inverted for 5 min then stained with haematoxylin stain for 20 min and washed 3 times with distilled water by putting the transwells in distilled water filled wells for 2 min then drying inverted for 5 min. Pictures

were taken for the stained cells in the basal surface using the fluorescence microscope (Leica DMRB, Wetzlar, Germany) under 40X objective lens magnification and the cells were directly counted.

The percent invasion was determined through the following equation:

$$Percent\ Invasion = \frac{Mean\ number\ of\ treated\ cells\ invading\ through\ coated\ permeable\ support\ membrane}{Mean\ number\ of\ untreated\ cells\ invading\ through\ coated\ permeable\ support\ membrane} \times 100$$

Results

Evaluation of compound toxicity using the MTT assay

The MTT cytotoxicity assay was performed in order to determine the IC_{50} values for the compound **(Figure 1)** to decide the non-toxic concentrations to be used in the 3D spheroid invasion assay. The IC_{50} is defined as the drug concentration that is required to cause 50% inhibition *in vitro.* The IC_{50} value of the compound was 69.2±25.5 µM.

Validation of the invasion assay using cRGDfV

Different concentrations of cRGDfV were tested to determine the effect of this compound on the inhibition of U87-MG cells invasion through collagen. cRGDfV was found to reduce the invasion of the U87-MG spheroids in a time and dose dependent manner **(Figure 2)**. The concentrations of the cRGDfV used including 100 nM, and 10 µM were selected based on the IC_{50} and IC_{10} values that were calculated based on the MTT chemo-sensitivity assay. Briefly the tested concentrations were selected to be not exceeding the IC_{50} and not less than the IC_{10} to avoid toxicity and guarantee efficiency.

Comparing the ability of the U87-MG cells to invade in the 3D invasion model with their invasion in 2D transwell invasion model

In an aim to validate the results of using 3D spheroid invasion assay to test the effect of the cRGDfV on the invasion of U87-MG cells, the transwell 2D invasion assay was optimised and used to test the effect of cRGDfV on the invasion of a monolayer of U87-MG cells. The results of both assays were plotted and the data was compared.

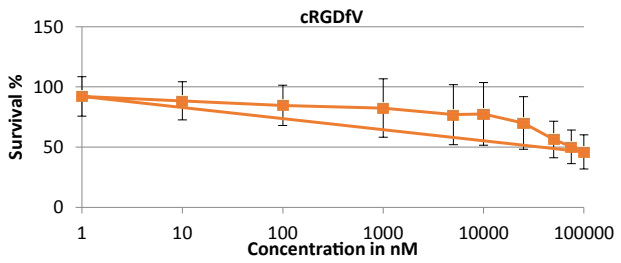

Figure 1 The results of the MTT cytotoxicity assay that performed on the U87-MG cells using the cRGDfV as a control. Results presented are an average of 3 independent experiments. The IC50 value was calculated using the proportionately calculation.

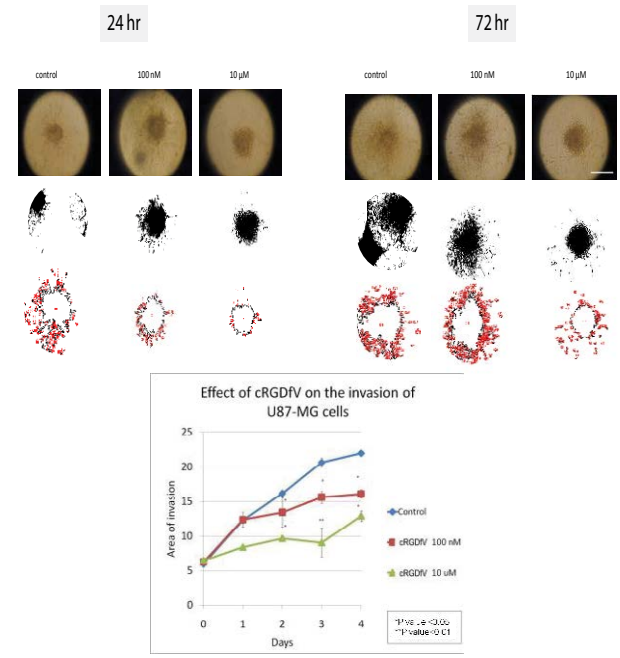

Figure 2 cRGDfV reduces U87-MG cells invasion in a concentration and time dependent manner. The pictures were taken over 4 days of invasion into collagen and analyzed by ImageJ (Scale bar: 100 µm).

Optimization of the 2D transwell invasion model to test the invasion of the U87-MG cells

First the seeding density of the U87-MG cells was determined through testing two different seeding densities including 5 × 10^4 cells/well and 5 × 10^5 cells/well. The cells were starved for 4 hrs under serum-free medium media before seeding them in the transwells. The cells showed high ability to invade through Matrigel coated transwells. The seeding density 5 × 10^4 cell/well was selected to be the better density and used for further work, because it resulted in clear difference between the invasions towards different concentrations of FCS as a chemo attractant compared to the other seeding density **(Figure 3)**.

The effect of cRGDfV on the ability of the U87-MG cells to invade in 2D transwell invasion model compared to its effect on their invasion in the 3D invasion model

Different concentrations of cRGDfV were used; the IC_{50} as obtained in the MTT cytotoxicity assay **(Figure 1)**, 100 fold less than the IC_{50} and 10 fold above the IC_{50}. cRGDfV showed a concentration dependent reduction in the U87-MG cell invasion through Matrigel coated transwells towards 10% FCS compared to the control untreated U87-MG cells **(Figure 4)**.

To compare the effect of cRGDfV on the invasion of the U87-MG cells through Matrigel coated 2D transwells with its effect on the invasion of U87-MG cells from 3D spheroids in collagen, the results of the two assays were plotted and the data was compared

that is stating the obvious. cRGDfV showed a significantly more potent effect on the invasion of U87-MG cells through Matrigel coated 2D transwells compared to its effect on the invasion of U87-MG cells from spheroids in collagen **(Figure 5)**.

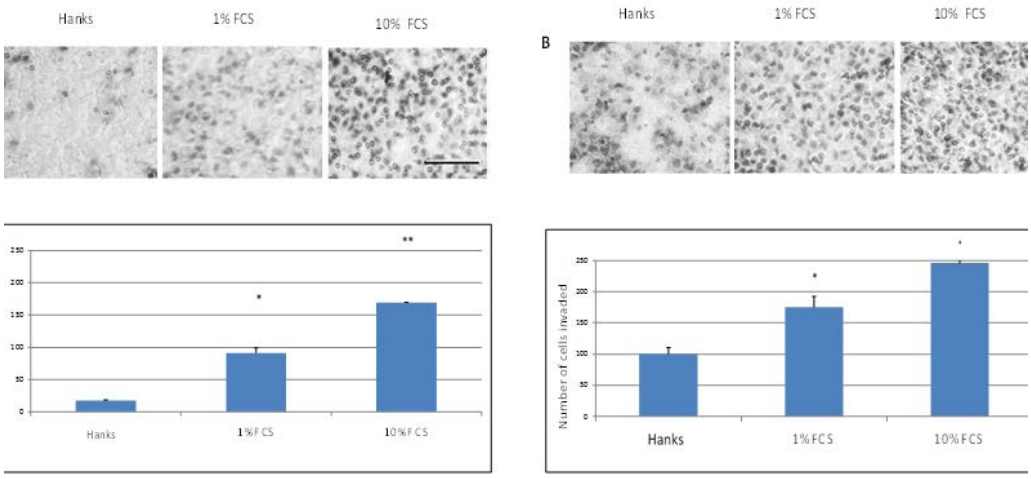

Figure 3 U87-MG cells invasion through Matrigel coated transwells (Scale bar: 100 µm). Two seeding densities were tested including. (A) 5 × 10^4 cell/well and (B) 1 × 10^5 cell/well to determine the best one to be used for further studies. The seeding density 5×104 cell/well was selected to be the optimum density, because a clear difference was detected between the invasion towards the 10% FCS media, 1% FCS media and Hanks balanced solution (**p<0.01, *p<0.05 (n=3)).

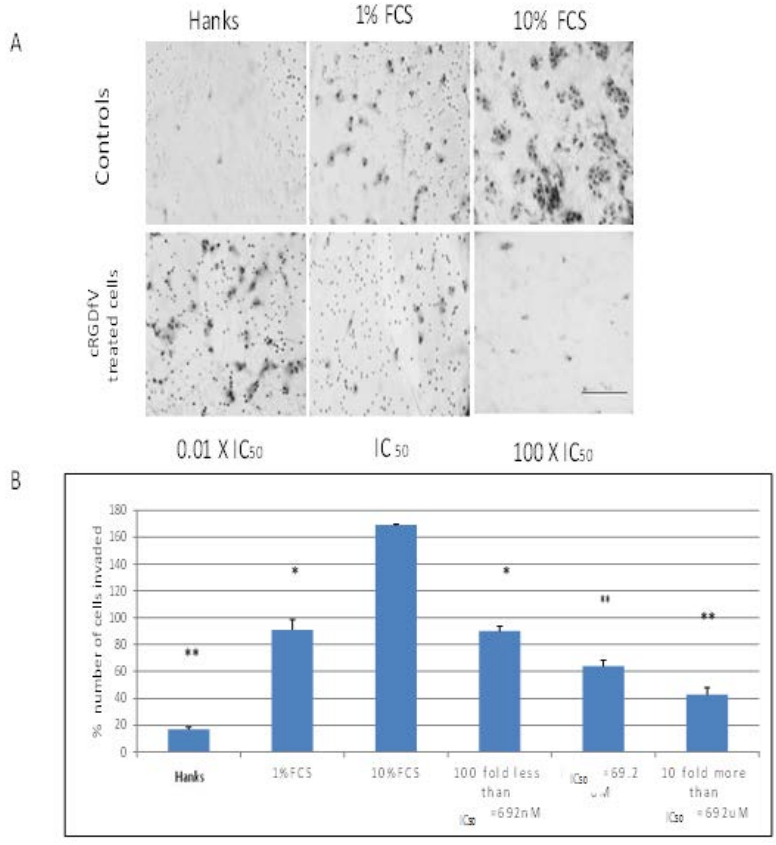

Figure 4 cRGDfV reduces cell invasion in a concentration dependent manner. (A) The pictures of the transwells after invasion of the untreated U87-MG cells towards different chemo attractants including 1% FCS, 10% FCS and hanks balanced solution (Scale bar: 100 µM). (B) Graphic representation shows a comparison between the invasion through transwells for the treated U87-MG cells and untreated controls (**p<0.01, *p<0.05 (n=3)).

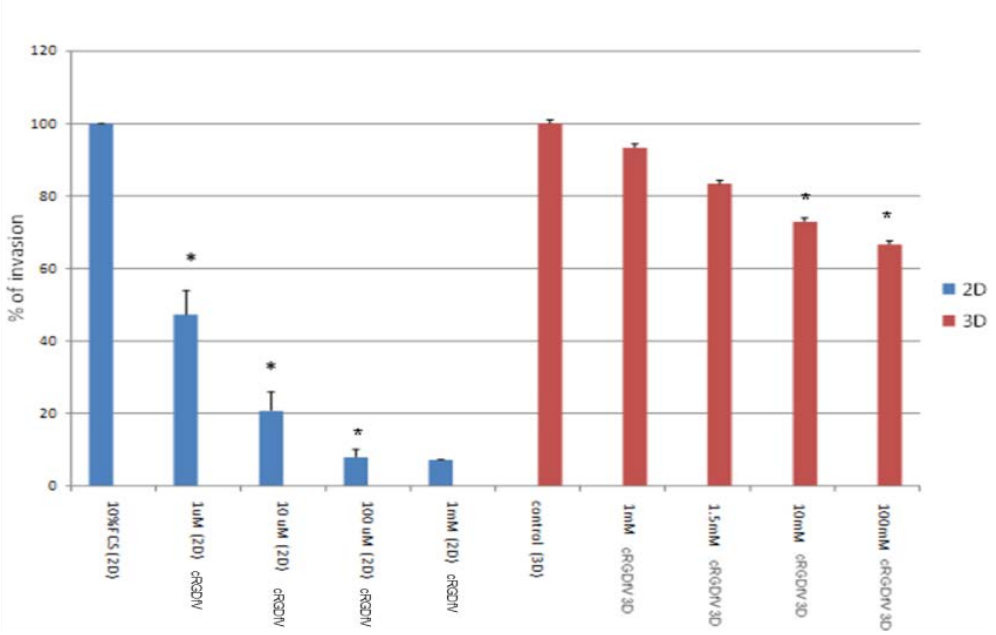

Figure 5 Comparison between the invasion of the U87-MG cells in the 2D transwell assay and there invasion in 3D spheroid assay under the effect of cRGDfV. cRGDfV was applied both in 2D and 3D models. Graphical presentation showing more potent effect for cRGDfV on the invasion of U87-MG cells through Matrigel coated 2D transwells compared to its effect on the invasion of U87-MG cells in 3D spheroid invasion assay. The chart shows a decrease in the percentage of invasion from 100% for the control to 7.2% for the cells treated with 1 mM of cRGDfV in the 2D transwells, compared to a much lower decrease in the invasion from 100% for the control to 67% for the spheroids treated with 100 mM of cRGDfV in the 3D spheroid invasion assay. 2D transwell controls of untreated U87-MG cells invading towards 10% FCS as chemoattractant and the 3D control was the untreated spheroids (**p<0.01, *p<0.05 (n=3)).

Discussion

The spheroid gel invasion assay was used in this study due to its ability to distinguish invasive cancer cells from non-invasive ones. The non-invasive cancer cells grow as compact spheroids with clear borders from the surrounding matrix, and no signs of invasion even after 7 days of culture [10]. On the other hand, invasive cells start to invade out from the embedded spheroids into the surrounding collagen as early as the first day after embedding [12]).

The spheroid gel invasion assay is ideal in giving an indication of the stromal cues that induce the invasive growth of cancer such as the interactions between the tumour cells and the stromal cells. Furthermore this assay maintains the structural and morphological properties of the cells allowing real-time monitoring and biochemical analysis of the basic features of the cellular invasion during the running time of the assay. Subsequently, computer analyses of images taken during the assay allow for calculating important parameters such as invasion area [9].

Collagen is the main interstitial matrix component in solid tumours, so it could be used in the invasion models to allow for easy observation and quantification in a close recapitulation of the *in vivo* situation [13]. Furthermore, collagen type I consists of native non-helical telopeptides situated at the N- and C-terminal ends, which are important in the formation of intermolecular covalent cross-links needed for the formation of the gel architecture that is considered as a barrier to tumour cell invasion. This makes the results obtained from this assay more reflective to the real drug delivery problems [14].

The 3D spherical invasion model is encouraging due to it is possible with this assay to get a good picture of what is going on in real time using live imaging compared to 2D transwell assays where this is harder to do. Furthermore, the cells invading into the collagen gels can be fixed using different fixation procedures and investigated using confocal microscopy.

The 2D transwell invasion assay was selected as a way to compare the results of the 3D invasion assay with the more commonly used 2D assay. In relation to the dose response effect, both the spheroid invasion assay and the transwell assays were comparable, although the transwell assay demonstrated a more potent effect. This result could be because the spheroid invasion assay may be more representative to the *in vivo* situation due to the presence of collagen which separates the compound and the cells compared to direct contact between the cells and the compound in the transwell assay. The presence of collagen mimics the extracellular matrix *in vivo* and forms as a physical barrier leading to a more realistic situation [15] compared to the transwells assay in which the cells are mixed as a suspension

with the compound [16]. Furthermore, the conformation of the U87-MG cells in a spheroid means that not all the cells are directly exposed to the compound and again contributes to explaining the higher resistance to treatment observed in the 3D invasion assay [8] compared to the 2D transwells in which the tumour cells present as suspension in direct contact with the treatment compound [11]. As a result of this, the spheroid invasion assay could be considered more reflective to the *in vivo*/clinical situation [17] where a drug must penetrate through layers of cells in the tumour tissue. The comparison between the 3D spheroid assay and the 2D transwell assay compared to the *in vivo* situation has been previously used as a tool for real and more detailed understanding of the compounds mechanisms of action. The same concentrations of GSK-3 inhibitors were able to cause higher level of inhibition of U87-MG cells invasion in the transwells compared to the 3D spheroid assay [18].

Studying the efficacy of a compound by two different methods such as the 3D invasion assay and the 2D transwells assay could help in characterizing a problem, if present, associated with compound penetration. For example if the compound didn't demonstrate a high effect when it was tested by the 3D invasion assay but in contrary it gave a high efficacy when tested by the 2D assay this could indicate that the compound does not penetrate tissue well, rather than having low affinity for the target. Consequently, the spheroid invasion assay could be able to give an idea about the problems associated with drug delivery and so could be considered closer to what could be seen in the clinic with this compound.

The limitation of this study is that the 3D spheroids could migrate instead of invading. This process could happen if the sphere attached to the plastic surface instead of being immersed in the collagen layers leading to wrong results. This limitation could be avoided by waiting enough time for the first collagen layer to polymerize before putting the spheres and putting the second collagen layer

Conclusion

In conclusion this work has demonstrated that cRGDfV showed a significantly more potent effect on the invasion of U87-MG cells through Matrigel coated 2D transwells compared to its effect on the invasion of U87-MG cells from spheroids in collagen. Furthermore, this work has demonstrated the importance of assay selection for a specific parameter, with results with potentially more relevance to the clinical situation with the 3D spheroid invasion assay compared to the 2D transwell assay.

Acknowledgment

We would like to thank the University of Jordan for providing funding. Thank you to all stuff in the ICT.

Conflict of Interest

There is no conflict of interest for any of the authors in this work.

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