Chapter 4

Evaluation of MM model

4.1 Introduction

Mesenchymal stem cells are a vital constituent of the BM microenvironment, where they provide essential support for the growth and differentiation of primitive haematopoietic cells as well as having the unique ability to self-renew and differentiate into multiple lineages including bone, cartilage, adipose and a variety of other connective tissues (Freidenstein *et al*., 1970 cited by Bianco *et al*., 2008; Janowska-Wieczorek *et al*., 2001). A dysfunction in this BM microenvironment contributes significantly to disease pathology, particularly in cancer (Ye *et al*., 2012; Bergfeld and DeClerck, 2010).

MM has been shown to recruit MSC through the release of various chemical signals, thus supporting the tumour cells enabling them to differentiate into a growing cancer (Corre *et al*., 2007). It has been well documented that MSC support the growth of MM cells by the release of IL-6 (Rosean *et al*., 2014; Arnulf *et al*., 2007; Cheung and Ness, 2002; Lokhorst *et al*., 1994) and that adhesion of the MM cell to MSC activates many pathways, resulting in up-regulation of cell cycle regulating proteins and anti-apoptosis proteins in the MM cell, ultimately leading to the development of drug resistance (Yang *et al*., 2003). Furthermore, these interactions lead to a decrease in osteoblast differentiation (Giuliani *et al*., 2005) with an increase in osteoclast activity (Dib *et al*., 2008) leading to bone destruction.

With chemotherapeutic agents targeting these MSC–MM interactions (Kim *et al*., 2015; Hideshema *et al*., 2001; Gupta *et al*., 2001), the failure of bone lesions to heal after response to therapy seems to support the idea of a permanent defect in BM-MSC in patients with MM (Nierste *et al*., 2014). However, the precise nature of the damage caused by chemotherapy and how interactions between MM cells and MSC impact on the functionality of the MSC are largely unknown and require further study.

The aim of this chapter is to evaluate the previously developed *in vitro* co-culture model (chapter 3) to study damage to HS5 in a MM setting. Physical characteristics and functional properties of HS5 cells were investigated in the presence or absence of U266B1 cells following exposure to the chemotherapeutic agents melphalan, thalidomide, lenalidomide, bortezomib and carfilzomib at the clinical dose. These were then compared to unexposed samples.

To examine the interaction of MM cells with the BM microenvironment three separate conditions were used: (1) independent cultures (MSC, HS5 and U266B1 cultured alone); (2) co-culturing MSC/HS5 and the MM cell line U266B1 without direct physical contact; (3) co-culturing the cells following exposure to one of the chemotherapeutic agents mentioned above to one cell compartment. Co-culturing previously chemotherapy treated cells with untreated cells will enable the investigation of a bystander effect and potentially aid in the understanding of the protection provided to MM cells from MSC (Xu *et al*., 2012; Markovina *et al*., 2010) and also whether this protection is afforded to MSC or whether MSC are altruistic in their protection of MM.

4.2 Methods

4.2.1 Trypan blue exclusion assay

Total cell numbers and cell viability was determined using the trypan blue exclusion assay after incubation with each of the chemotherapeutic agents at 1, 16 (for melphalan), 24 (for every other drug except melphalan), 48 and 72 hrs post exposure. Cells were sampled at 16 hrs post melphalan exposure as opposed to 24 hrs, as this was previously noted by Spanswick *et al*. (2002) to be the optimum time for DNA crosslink formation in patient myeloma plasma cells. This is further outlined in section 2.3.2.

4.2.2 Microscopy

Phase contrast microscopy of HS5 stromal cells was used to visualise cells immediately prior to treatment and at 1, 16 (for melphalan), 24 (for every

other drug except melphalan), 48 and 72 hrs time points post exposure of drug (see section 2.4.1).

4.2.3 Differentiation of HS5

The capacity of HS5 stromal cells to differentiate into osteogenic and adipogenic lineages following exposure to chemotherapy for 1 hr and while in co-culture with U266B1 cells was investigated (see section 2.7.1).

4.2.4 Flow Cytometry

HS5 stromal cells were assessed for the cell-surface expression of CD45, CD73, CD105, CD34 and CD14 at 72 hrs following treatment with chemotherapeutic agents for 1 hr (see section 2.7.2).

4.2.5 ELISA

In order to measure IL-6 released from the MSC, HS5 and U266B1 cell lines following co-culture and exposure to chemotherapeutics for 1 hr, an in-house sandwich ELISA was developed and utilised (see section 2.6). Samples were measured for IL-6, 72 hrs post exposure to drug.

4.2.6 Statistical Analysis

All results in this chapter are presented as means ± SD of at least three independent experiments with number of replicates stated in respective experiments. Statistical comparisons were performed by an unpaired Student's t-test for all results using Microsoft Excel, Differences between means were considered statistically significant if the p value was less than 0.05.

4.3 Results

4.3.1 Trypan blue assessment of primary MSC, HS5 and U266B1 cell lines following chemotherapy

The cytotoxic effects of chemotherapeutic agents used in MM treatment were assessed using the *in vitro* model developed in chapter 3. Initially primary MSC were used in the model when studying the effects of melphalan and compared with the stromal cell line HS5 in order to understand the changes in this constituent of the BM microenvironment during MM progression. Furthermore when studying the effects of melphalan, resistant U266B1 cells were also used within the model and compared with normal U266B1 cells. The total cell numbers and viability of primary MSC, HS5 stromal and U266B1 cell lines following chemotherapy at each clinical dose was compared. Cell counts were taken at 1, 16 (for melphalan) 24 (for all agents except melphalan), 48 and 72 hrs post exposure to chemotherapy. For cells exposed to melphalan, cell counts were taken at 16 hrs post exposure rather than 24 hrs as this was previously noted as the optimum time for DNA crosslinks to be formed in MM cells (Spanswick *et al*., 2002) and samples were also taken for genotoxic analysis (chapter 5). Each of the chemotherapeutic agents investigated has been presented in individual sections for ease of reading. Data was analysed using Microsoft excel software with the mean of three independent experiments used to create the graphs.

4.3.1.1 Melphalan

Primary MSC, HS5 stromal cells, U266B1 sensitive and U266B1 melphalan resistant were exposed to melphalan (32.8 µM) for 1 hr either alone or in co-culture (via an insert). Samples were taken immediately (1 hr), 16, 48 and 72 hrs after exposure.

4.3.1.1.1 MSC cultured alone and exposed to melphalan

Firstly looking at the effects of melphalan on primary MSC; when cultured alone after exposure to melphalan there was an increase in their total cell

numbers at 16 hrs ($p<0.05$) from 1 hr (figure 4.1). Total cell numbers then reduced at 48 hrs and were significantly lower than the untreated ($p<0.05$). Cell numbers then increased significantly at 72 hrs ($p<0.05$). Although total cell numbers increased, MSC viability declined at each time point post exposure to melphalan and was significantly reduced at 72 hrs ($p<0.001$) compared to the untreated. MSC not exposed to melphalan had an increase in cell numbers at each time point and had a constant viability above 70%.

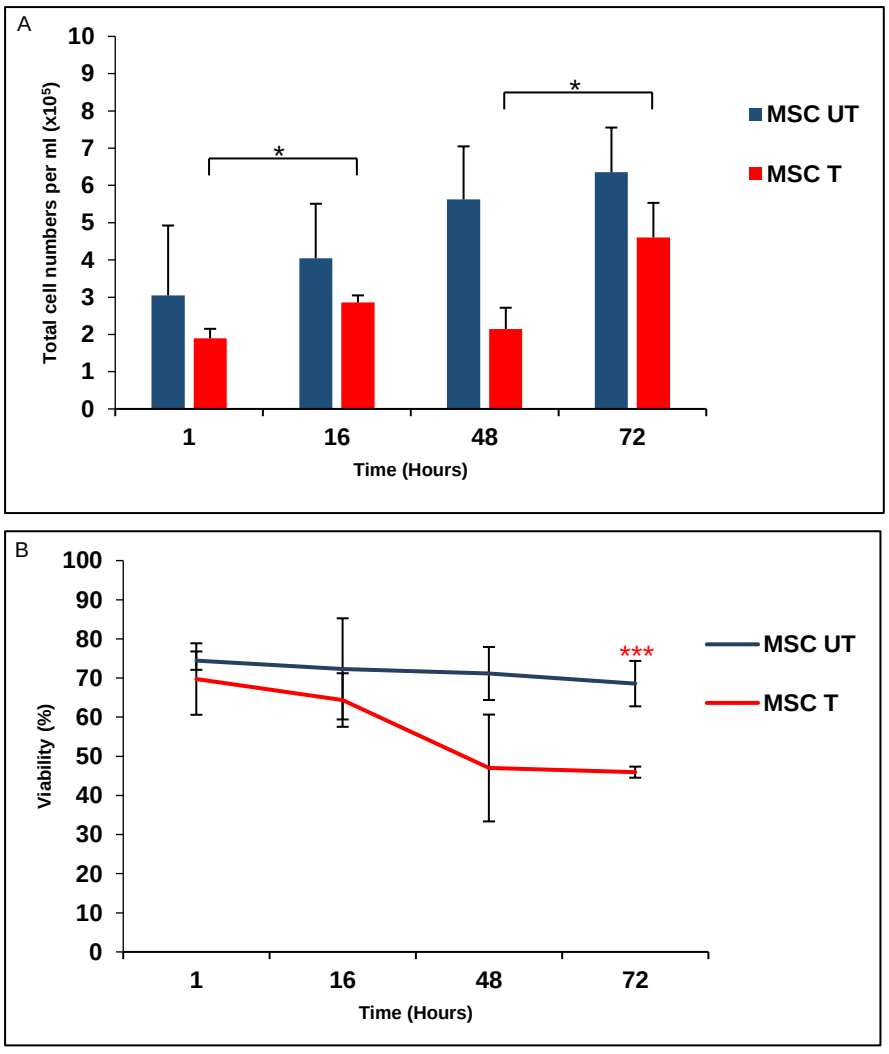


Figure 4.1 Total cell numbers (A) and viability (B) of MSC after exposure to melphalan when cultured alone. MSC initially seeded at 2.0 x 10^4 cells / cm^2 and cultured alone were exposed to 32.8 µM melphalan for 1 hr. MSC total cell numbers fluctuated over the 72 hrs following exposure to melphalan. Cell viability declined at each time point following melphalan treatment. Cell viability was measured at 1, 16, 48 and 72 hrs post exposure. MSC T: Mesenchymal stem cell treated; MSC UT: Mesenchymal stem cell untreated. Bars represent mean ± SD(n=3). Significant differences between treated with untreated is indicatd with a red * (* $p<0.05$, *** $p<0.001$).

4.3.1.1.2 U266B1 cultured alone and exposed to melphalan

As illustrated in figure 4.2, melphalan produced a notable time dependent cytotoxic effect on U266B1 cells when cultured alone. U266B1 cell numbers steadily decreased over 72 hrs post exposure to melphalan compared to U266B1 cells not exposed to drug. Total cell numbers were significantly reduced at 48 ($p<0.05$) and 72 hrs ($p<0.01$) post exposure compared to the untreated. This finding correlates with the viability of these cells which significantly decreased at 16 ($p<0.01$), 48 ($p<0.05$) and 72 hrs ($p<0.01$) compared to the untreated control.

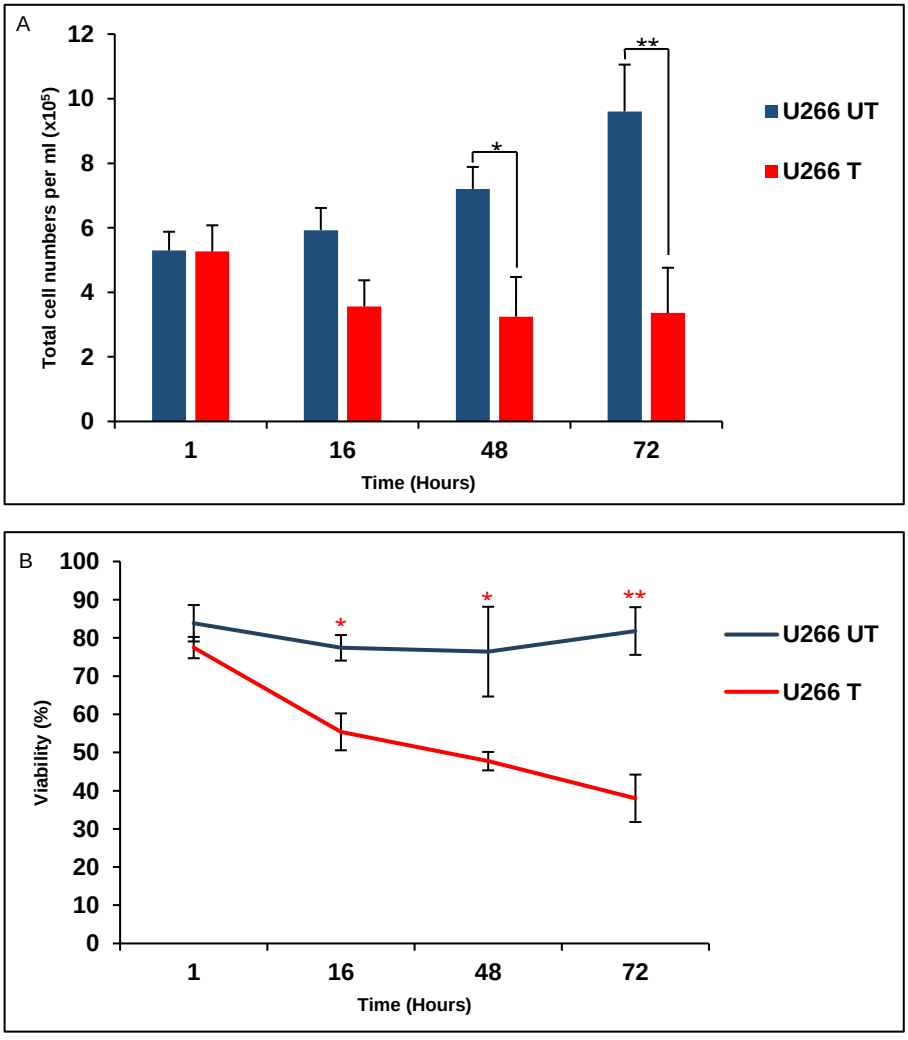


Figure 4.2 Total cell numbers (A) and viability (B) of U266B1 cells after exposure to melphalan when cultured alone. U266B1 cells, initially seeded at 5 x 10^5 cells in 1 ml of medium were cultured alone and exposed to 32.8 µM melphalan for 1 hr. Melphalan caused a reduction in the total cell numbers and viability of U266B1 cells at each time point post exposure. Cell counts were measured at 1, 16, 48 and 72 hrs post exposure. U266 T: U266B1 melphalan treated; U266B1 UT: U266B1 untreated. Bars represent mean ± SD. Significant differences between treated with untreated is indicatd with a red * (* $p<0.05$, ** $p<0.01$) (n=3).

4.3.1.1.3 MSC co-cultured with U266B1 and exposed to melphalan

When MSC were co-cultured with U266B1 cells and separated by an insert, it had a deleterious effect on their viability and proliferation (figure 4.3). MSC that were not exposed to drug experienced a reduction in cell numbers up to 48 hrs before improving at 72 hrs. Total cell numbers at 72 hrs however were lower than that seen in independent MSC culture (figure 4.1). At 1 hr post exposure to melphalan, MSC co-cultured with U266B1 had a reduction in total cell numbers compared to the control ($p<0.05$) which improved slightly at 16 hrs but reduced further at 48 hrs. There was a significant improvement in total cell numbers at 72 hrs in both MSC exposed to melphalan ($p<0.05$) and the untreated control ($p<0.05$). Cell viability of MSC in co-culture with U266B1 decreased at each time point and this was further exacerbated by melphalan treatment which caused a significant reduction in viability at 72 hrs compared to the untreated ($p<0.05$). Although total cell numbers had increased at 72 hrs, viability was below 30% in cells previously exposed to melphalan. These findings indicate U266B1 cells exacerbate the cytotoxic effect of melphalan on MSC without the need for direct contact.

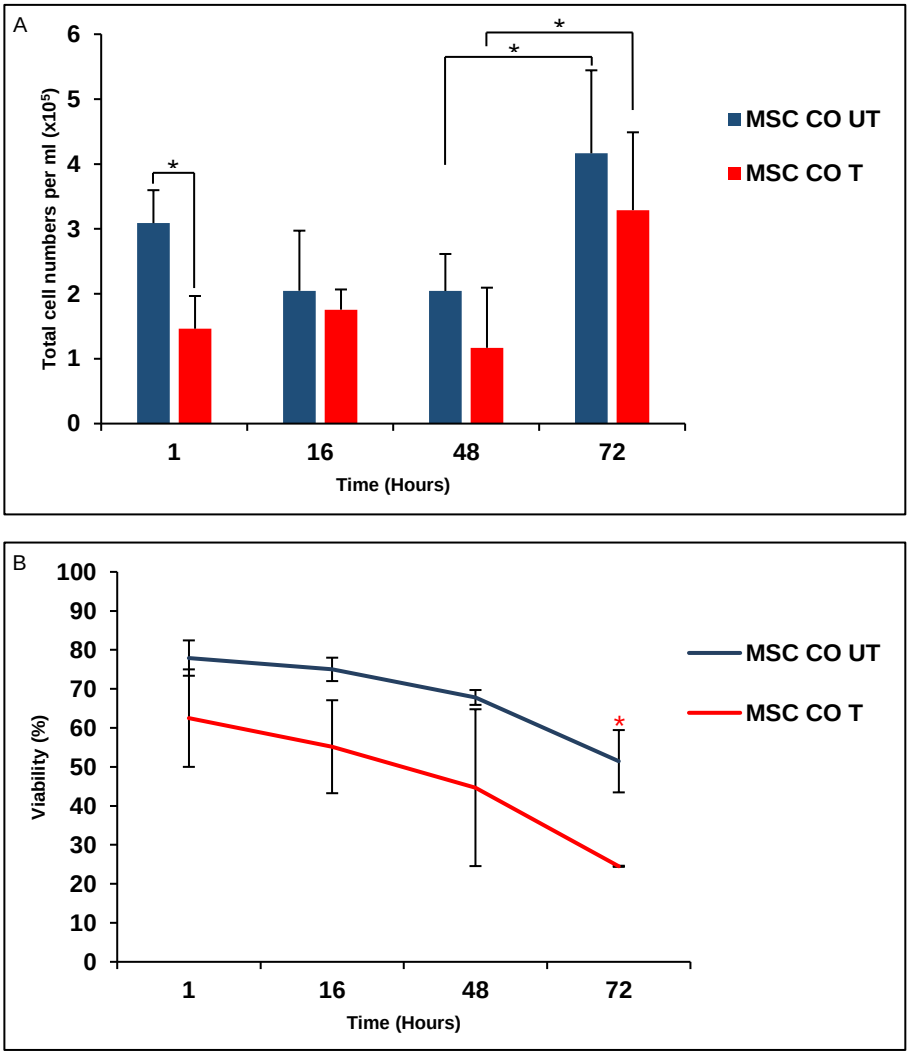


Figure 4.3 Total cell numbers (A) and viability (B) of MSC after exposure to melphalan when in co-culture with U266B1 cells. MSC (2.0 x 10^4 cells / cm^2) and U266B1 (5 x 10^5 cells / ml) were cultured together via an insert and exposed to 32.8 µM melphalan for 1 hr. Total cell numbers of MSC increased between 48 and 72 hrs post exposure to melphalan. Co-culture of these cells caused a reduction in viability in the MSC which was further exacerbated by melphalan treatment. Cell counts were measured at 1, 16, 48 and 72 hrs post exposure. MSC CO T: Mesenchymal stem cell treated in co-culture with U266B1; MSC CO UT: Mesenchymal stem cell in co-culture with U266B1 untreated. Bars represent mean ± SD. Significant differences between treated with untreated is indicated with a red * (* $p<0.05$) (n=3).

4.3.1.1.4 U266B1 co-cultured with MSC and exposed to melphalan

When U266B1 were treated in a co-culture with MSC (figure 4.4), total cell numbers did not decrease as opposed to those exposed to melphalan alone (figure 4.2), suggesting a possible protective effect of MSC on U266B1 cells. In contrast to the MSC compartment (figure 4.3), U266B1 cell numbers (although lower than the untreated) increased at each time point following exposure to melphalan. However, total cell numbers were significantly lower than the untreated control at 16 ($p<0.05$) and 72 hrs ($p<0.05$) post exposure.

Viability of U266B1 cells untreated and those exposed to melphalan decreased at each time point with melphalan treated U266B1 significantly decreased at 16 hrs ($p<0.05$) compared to the control. However, viability was not significantly reduced at subsequent time points and was also higher than the viability of the MSC that were in co-culture with the U266B1 (figure 4.3). These findings suggest MSC are protecting U266B1 from the effects of melphalan.

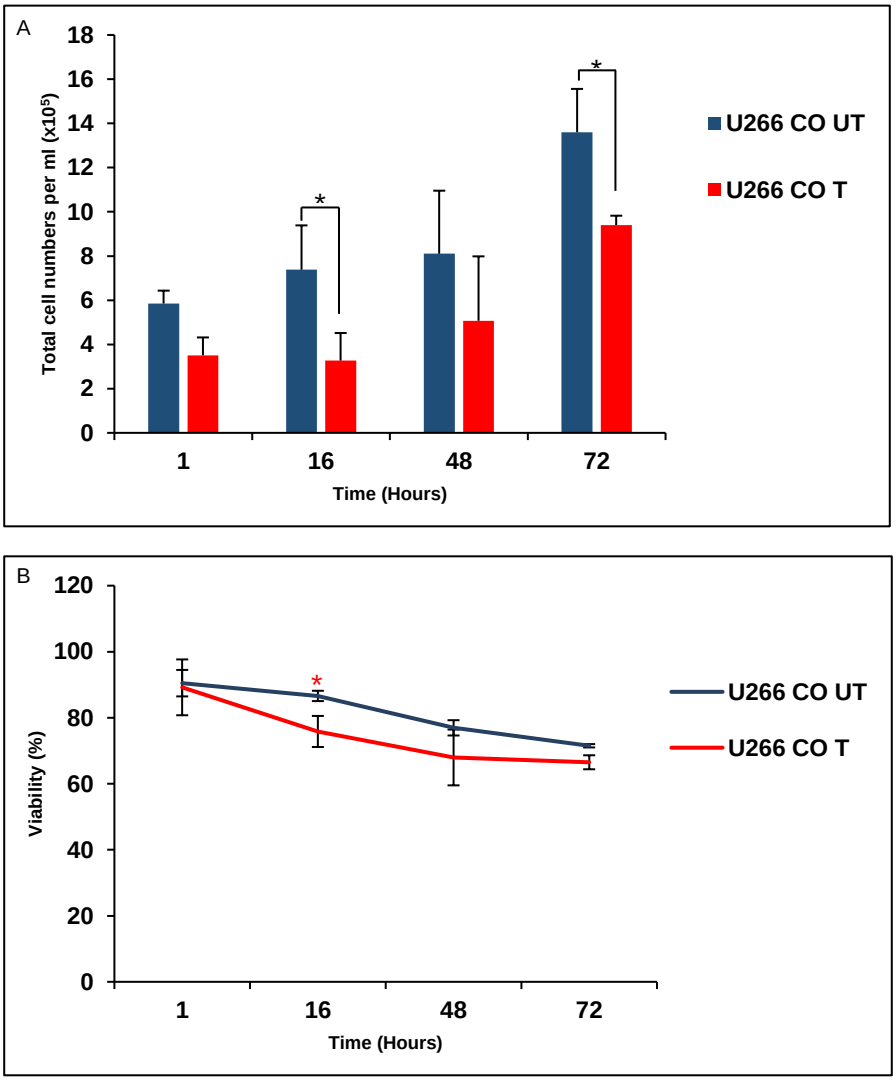


Figure 4.4 Total cell numbers (A) and viability (B) of U266B1 cells after exposure to melphalan when in co-culture with MSC. MSC and U266B1 were cultured together via an insert and both cell types were exposed to 32.8 µM melphalan for 1 hr. Total cell numbers and viability of U266B1 cells when cultured with MSC and exposed to melphalan is improved as opposed to when cultured alone (figure 4.2). U266B1 cell counts were measured at 1, 16, 48 and 72 hrs post exposure. U266B1 CO T: U266B1 treated in co-culture with MSC; U266B1 CO UT: U266B1 untreated in co-culture with MSC. Bars represent mean ± SD. Significant differences between treated with untreated is indicated with a red * (* $p<0.05$) (n=3).

4.3.1.1.5 MSC bystander model

To investigate whether a bystander effect occurs between these two cell types, MSC and U266B1 respectively were cultured separately and treated with melphalan before being co-cultured with previously untreated U266B1 or MSC. Total cell numbers of MSC that were treated with melphalan and cultured with untreated U266B1 in comparison to MSC that were untreated and cultured with treated U266B1 did not differ significantly (figure 4.5).

There was a reduction in total cell numbers of treated MSC at 48 hrs before an increase at 72 hrs post exposure. MSC that were treated with melphalan and then cultured with unexposed U266B1 cells had a fluctuation in viability over the 72 hr period. There was a significant decrease in treated MSC viability at 16 hrs ($p<0.01$) which improved at 48 hrs but again decreased at 72 hrs post exposure.

MSC that were untreated and co-cultured with treated U266B1 had a significant improvement in total cell numbers between 48 and 72 hrs ($p<0.05$). Viability of MSC that were not exposed to drug and cultured with U266B1 decreased at 16 and 48 hrs, before reaching a plateau at 72 hrs.

These findings do not definitively indicate a bystander effect between these two cell types. However, the improvement in viability of MSC that were exposed to melphalan may be related to their interactions with the untreated U266B1 cells. Conversely the decrease in MSC viability not exposed to melphalan directly may be a consequence of the interactions with the treated U266B1 cells.

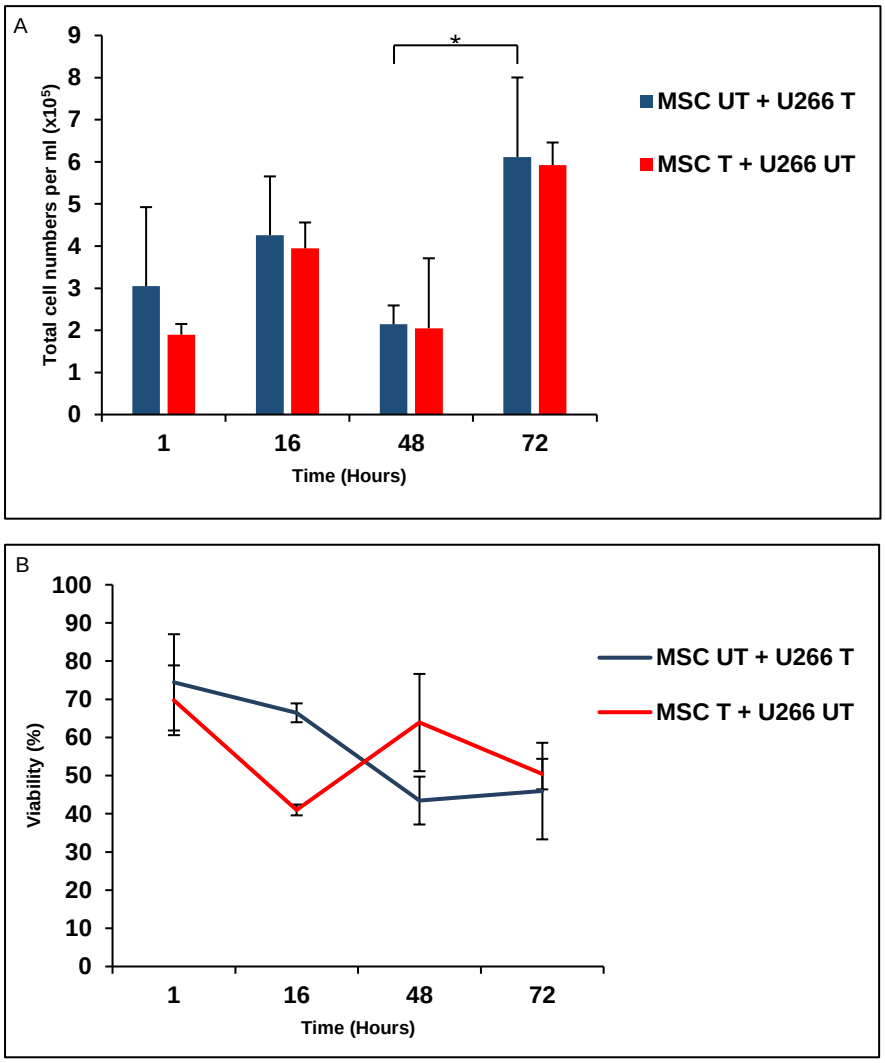


Figure 4.5 Total cell numbers (A) and viability (B) of MSC after exposure to melphalan either directly or when in culture with previously exposed U266B1 cells. MSC (2 x 10^4 cells / ml) / U266B1 (5 x 10^5 cells / ml) were cultured alone and exposed to 32.8 µM melphalan for 1 hr and then co-cultured with untreated U266B1/MSC respectively with separation (insert). Cell counts were measured at 1, 16, 48 and 72 hrs post exposure. MSC T + U266B1 UT: Mesenchymal stem cell treated before co-culture with untreated U266B1 cells; MSC UT + U266B1 T: Mesenchymal stem cells untreated before co-culture with treated U266B1 cells. Bars represent mean ± SD. Significant differences between cells are indicated with a * (*$p<0.05$) (n=3).

4.3.1.1.6 U266B1 bystander model

Total cell numbers of U266B1 cells that were not exposed to melphalan and cultured with previously melphalan exposed MSC, remained relatively constant at 1 and 16 hrs post exposure, before a significant increase at 48 hrs ($p<0.01$) which increased further at 72 hrs (figure 4.6, A). Viability of untreated U266B1 cells also improved between 48 and 72 hrs (figure 4.6, B).

U266B1 cells treated with melphalan and cultured with untreated MSC had similar total cell numbers at 1, 16 and 48 hrs post exposure before a significant increase at 72 hrs ($p<0.05$). There was a reduction in viability of U266B1 cells that had been previously exposed to melphalan at 48 hrs that then improved at 72 hrs. These findings suggest that the early cytotoxic effects of melphalan are reversed when in culture with MSC indicating a possible bystander effect. Where MSC viability was noted to decrease at 72 hrs (figure 4.5) U266B1 cell viability improved.

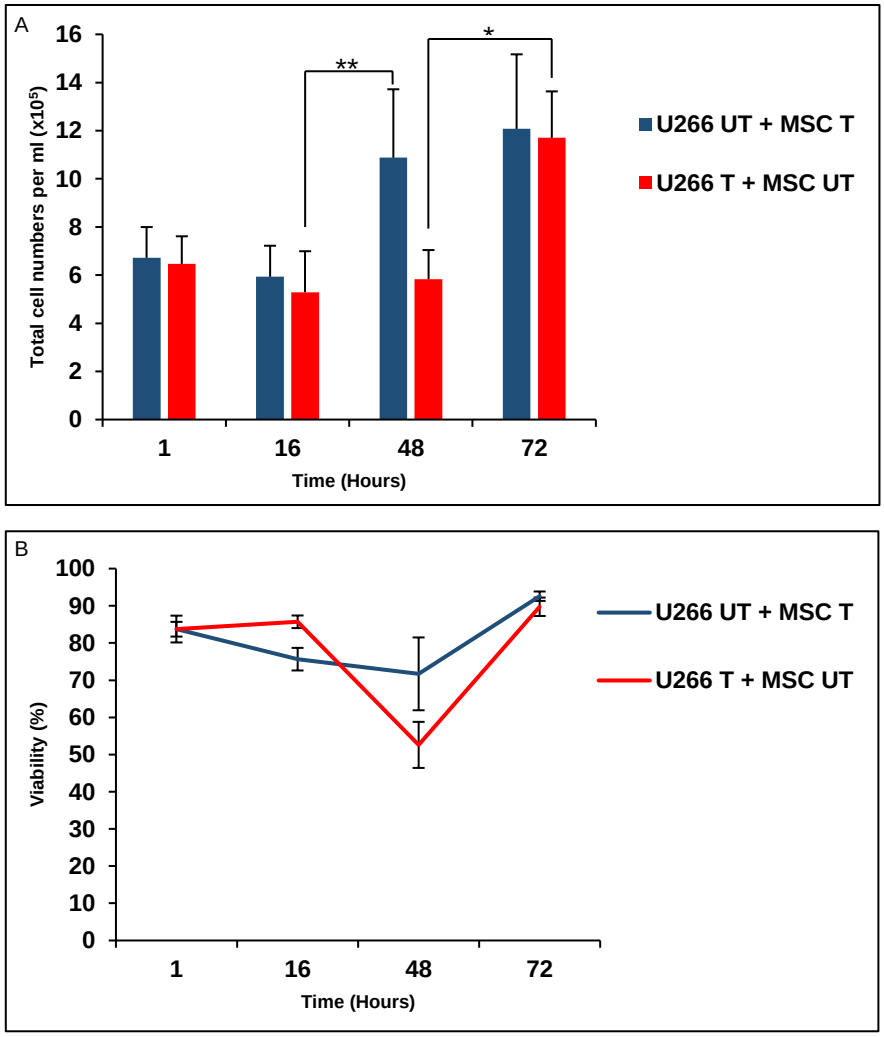


Figure 4.6 Total cell numbers (A) and viability (B) of U266B1 cells after exposure to melphalan either directly or when in culture with previously exposed MSC. U266B1 (5 x 10^5 cells / ml) were either cultured alone and exposed to 32.8 µM melphalan for 1 hr or left untreated. These cells were then co-cultured (via an insert) with either untreated or melphalan treated MSC. Cell counts were recorded at 1, 16, 48 and 72 hrs post exposure. U266B1 T + MSC UT: U266B1 treated cultured with MSC untreated; U266B1 UT + MSC T: U266B1 untreated cultured with treated MSC. Bars represent mean ± SD. Significant differences between cells are indicated with an * (* $p<0.05$, ** $p<0.01$) (n=3).

4.3.1.1.7 HS5 cells cultured alone and exposed to melphalan

HS5 cells cultured alone after exposure to melphalan had reduced cell numbers, compared to the control at each time point with a significant reduction in total cell numbers at 48 ($p<0.05$) and 72 hrs ($p<0.05$) (figure 4.7). As seen when primary MSC were cultured alone, viability of HS5 cells was reduced at each time point. HS5 viability post exposure to melphalan was significantly reduced at 48 hrs ($p<0.001$) and 72 hrs ($p<0.001$) compared to the untreated control. HS5 viability at 72 hrs was similar to that of primary MSC with both cell types having viability bewteen 40 and 50%.

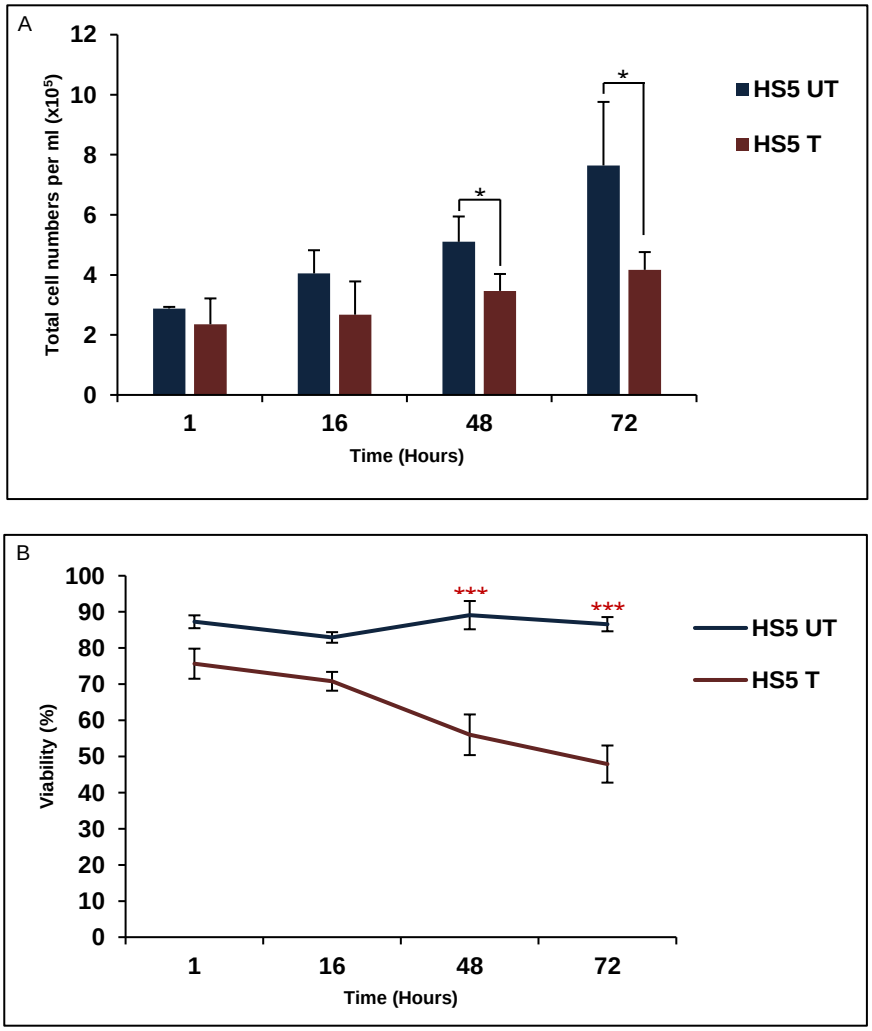


Figure 4.7 Total cell numbers (A) and viability (B) of HS5 cells after exposure to melphalan when cultured alone. HS5 cultured alone and exposed to melphalan (32.8 µM) for 1 hr experienced a significant decrease in total cell numbers and viability. Cell counts were measured at 1, 16, 48 and 72 hrs post exposure. HS5 T: HS5 cell treated; HS5 UT: HS5 cell untreated. Bars represent mean ± SD. Significant differences between treated with untreated is indicatd with a red * (** $p<0.05$, ***$p<0.001$) (n=3).

4.3.1.1.8 Melphalan resistant U266B1 cultured alone and exposed to melphalan

Melphalan resistant U266B1 cells were cultured alone and exposed to melphalan. For comparison, U266B1 sensitive cells were also cultured alone and exposed to melphalan. As illustrated in figure 4.8, melphalan resistant U266B1 cells exposed to melphalan, increase in number at each time point and have an improved viability compared to sensitive U266B1 exposed to melphalan when cultured alone. At 16 hrs post exposure both U266B1 sensitive and melphalan resistant cells have significantly lower total cell numbers compared to their untreated control ($p<0.01$). U266B1 sensitive cells have significantly lower total cell numbers at 48 ($p<0.001$) and 72 hrs ($p<0.01$) compared to the untreated. U266B1 melphalan resistant cells also have significantly lower total cell numbers at 48 ($p<0.05$) and 72 hrs ($p<0.01$) compared to the untreated. However at 48 hrs post exposure to melphalan, melphalan resistant cells have significantly higher cell numbers compared to U266B1 sensitive cells ($p<0.01$) that were exposed to drug, which was also significant at 72 hrs post exposure ($p<0.05$). Viability of the U266B1 sensitive cells significantly decreased at 16 ($p<0.01$), 48 ($p<0.05$) and 72 hrs ($p<0.01$) compared to the melphalan resistant cells. The cytotoxic effects of melphalan on U266B1 sensitive cells was as expected and similar to that in figure 4.2. Melphalan resistant U266B1 cells had improved proliferation and viability compared to the sensitive U266B1 cells when exposed to melphalan.

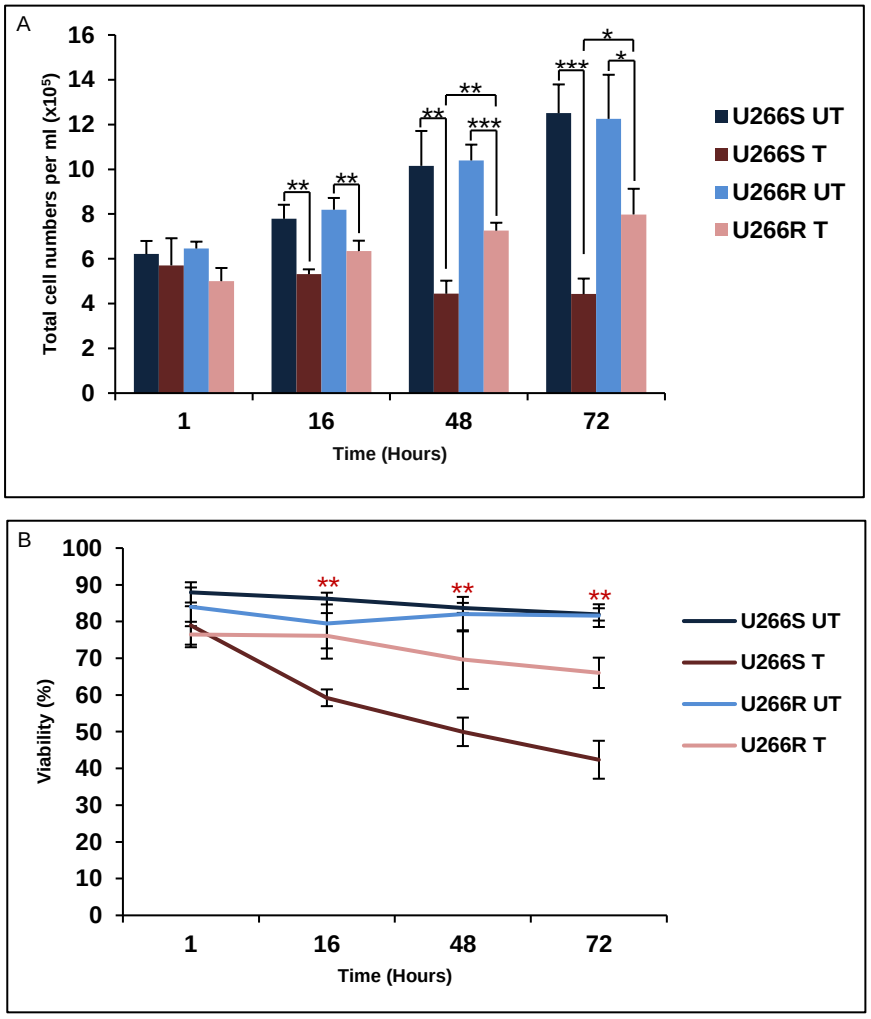


Figure 4.8 Total cell numbers (A) and viability (B) of melphalan resistant U266B1 compared to non-resistant (sensitive) U266B1 cells following exposure to melphalan and cultured alone. U266B1 resistant and sensitive were cultured alone separately and exposed to melphalan (32.8 µM) for 1 hr. Melphalan resistant U266B1 cells had significantly improved cell numbers and viability compared to U266B1 sensitive cells. Cell counts were measured at 1, 16, 48 and 72 hrs post exposure. Abbreviations: U266S UT: U266B1 sensitive untreated; U266B1S T: U266B1 sensitive treated; U266B1R UT: U266 melphalan resistant untreated; U266B1R T: U266B1 melphalan resistant treated. Bars represent mean ± SD. Significant differences between treated with untreated is indicatd with a red * Significant differences between cells are indicated with an * (* $p<0.05$, ** $p<0.01$, ***$p<0.001$) (n=3).

4.3.1.1.9 HS5 cells co-cultured with U266B1 sensitive and melphalan resistant U266B1 cells

HS5 cells were co-cultured with melphalan resistant and sensitive U266B1 cells and exposed to melphalan while in co-culture (figure 4.9). Both HS5 cells cultured with melphalan resistant U266B1 and U266B1 sensitive cells had increased cell numbers at each time point when not exposed to melphalan. Viability of these cells did not differ significantly when left untreated.

Melphalan exposure resulted in a failure of HS5 cells co-cultured with U266B1 sensitive cells to increase in number at each time point and had significantly lower total cell numbers than the untreated at 48 ($p<0.001$) and 72 hrs ($p<0.01$). HS5 cultured with melphalan resistant U266B1 cells and exposed to melphalan had a significant decrease in total cell numbers at 48 ($p<0.05$) and 72 hrs ($p<0.01$) post exposure compared to the untreated control.

Cell viability of HS5 co-cultured with either U266B1 melphalan resistant or U266 sensitive cells after melphalan exposure decreased at each time point. Both HS5 cells co-cultured with melphalan resistant and sensitive U266B1 cells had significantly reduced viability at 72 hrs compared to each untreated co-culture ($p<0.001$). Collectively these findings suggest that irrespective of the sensitivity or resistance of the MM cells to melphalan HS5 cells are not protected from the cytotoxic effects of melphalan when in co-culture.

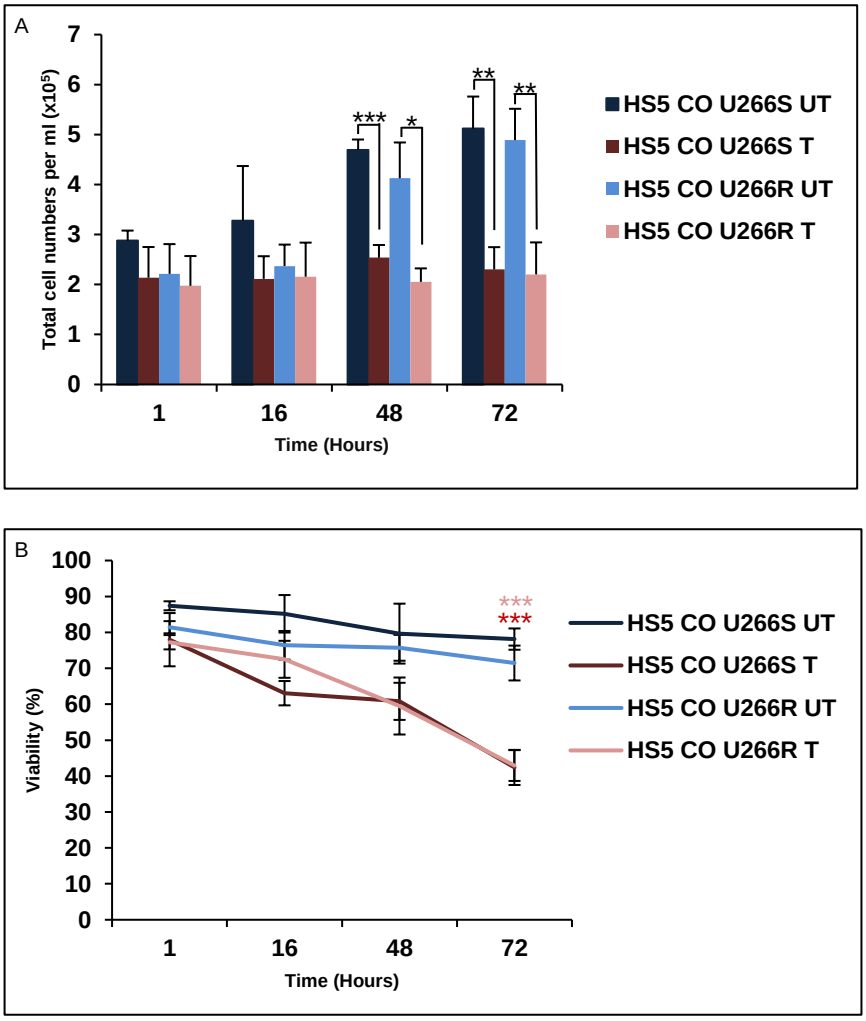


Figure 4.9 Total cell numbers (A) and viability (B) of HS5 cells after exposure to melphalan in co-culture with melphalan resistant U266B1 cells. HS5 cells co-cultured with melphalan resistant U266B1 via an insert were exposed to melphalan (32.8 µM) for 1 hr. Both melphalan resistant and melphalan sensitive co-culture with HS5 resulted in significant cytotoxic effects as a result of melphalan treatment. Cell counts were recorded at 1, 16, 48 and 72 hrs post exposure. Abbreviations: HS5 CO U266B1S UT: HS5 co-cultured with U266B1 sensitive cells and untreated; HS5 CO U266B1S T: HS5 co-cultured with U266B1 sensitive cells and treated; HS5 CO U266B1R UT: HS5 co-cultured with U266B1 melphalan resistant cells and untreated; HS5 CO U266B1R T: HS5 co-cultured with U266B1 melphalan resistant cells and treated. Bars represent mean ± SD Significant differences between treated with untreated is indicated with *. Red * when compared with U266S, pink * when compared to U266R (*p<0.05, **p<0.01, *** p<0.001) (n=3).

4.3.1.1.10 Melphalan resistant U266B1 cells in co-culture with HS5 cells

Melphalan resistant U266B1 and sensitive U266B1 cells were co-cultured with HS5 cells and exposed to melphalan when in co-culture. Melphalan resistant U266B1 cells total cell numbers increased at each time point with viability consistently above 70% over the 72 hr period post exposure to melphalan (figure 4.10). Melphalan resistant cells that were exposed to melphalan in co-culture with HS5 had significantly lower cell numbers at 72 hrs compared to the untreated.

U266B1 sensitive cells that were exposed to melphalan in co-culture with HS5 had significantly lower cell numbers at 16 ($p<0.05$), 48 ($p<0.05$) and 72 hrs ($p<0.05$) compared to the untreated. There were significantly more melphalan resistant cells than U266B1 sensitive cells at 72 hrs following exposure to melphalan. Viability of the melphalan treated U266B1 sensitive cells was significantly decreased at 72 hrs ($p<0.05$) as opposed to melphalan resistant cells. However viability was not as low as when exposed to melphalan in independent culture. This demonstrates that both melphalan resistant and sensitive U266B1 cells are protected from the cytotoxic effects of melphalan when co-cultured with HS5 cells.

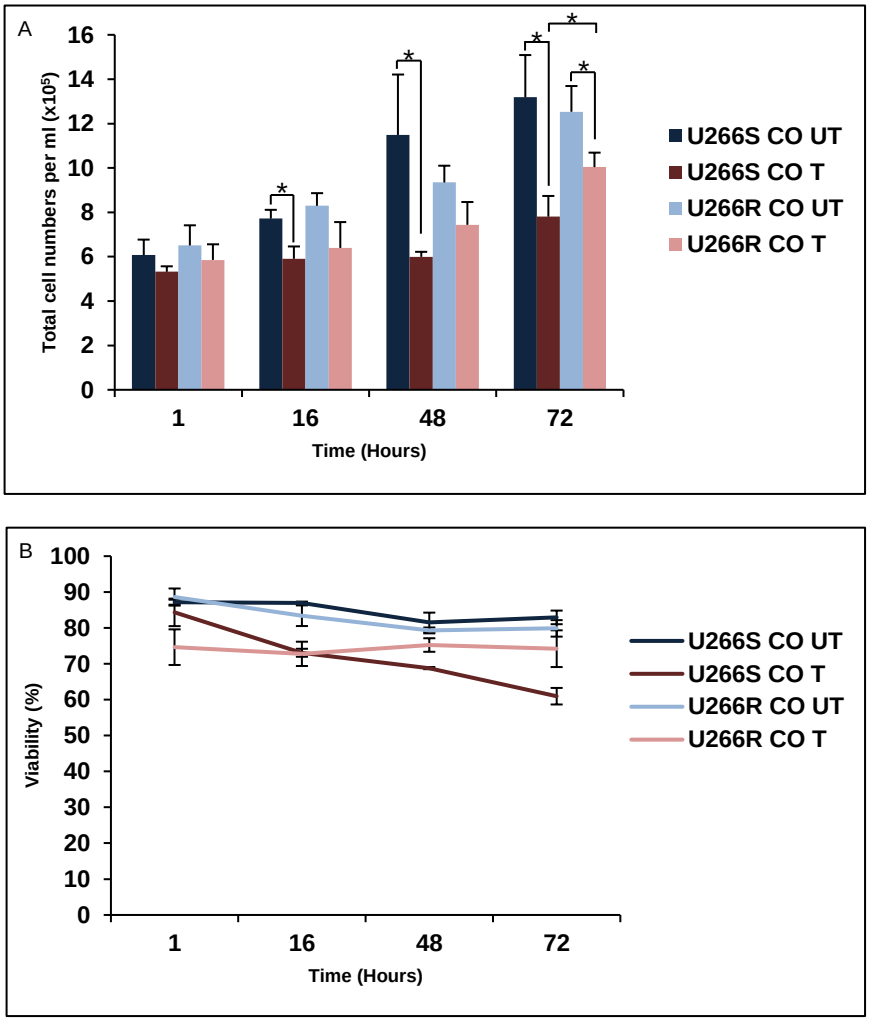


Figure 4.10 Total cell numbers (A) and viability (B) of U266B1 sensitive and melphalan resistant U266B1 after exposure to melphalan when in co-culture with HS5 cells. HS5 and melphalan resistant U266B1 cells were co-cultured via an insert and exposed to melphalan (32.8 µM) for 1 hr. Melphalan resistant U266B1 cells had improved cell numbers and viability compared to U266B1 sensitive cells following exposure to melphalan when in co-culture with HS5. Cell counts were measured at 1, 16, 48 and 72 hrs post exposure. Abbreviations: U266B1S CO UT: U266B1 sensitive cells co-cultured with HS5 untreated; U266B1S CO T: U266 sensitive cells co-cultured with HS5 and treated; U266B1R CO UT: U266B1 melphalan resistant cells co-cultured with HS5 and untreated; U266B1R CO T: U266B1 melphalan resistant co-cultured with HS5 and treated. Bars represent mean ± SD Significant differences between cells are indicated with a * (*p<0.05, **p<0.01, *** p<0.001)) (n=3).

4.3.1.2 Immunomodulatory agents

Subsequent experiments studying the cytotoxic effects of immunomodulatory agents and proteasome inhibitors were carried out using the stromal cell line HS5 and U266B1 sensitive cells. HS5 stromal cells and U266B1 cells were exposed to either thalidomide (200 ng / ml) or lenalidomide (4 μM) for 1 hr either alone or in co-culture (via an insert). Samples were taken immediately (1 hr), 24, 48 and 72 hrs after exposure.

4.3.1.2.1 HS5 cells cultured alone treated with thalidomide or lenalidomide

Treatment of HS5 cells with thalidomide did not result in a significant decrease in total cell numbers compared to the control (figure 4.11). Viability of HS5 cells was significantly reduced at 1 hr post exposure ($p<0.01$) compared to the control. However HS5 cell viability improved at each time point thereafter and was not significantly different to the control at 72 hrs ($p>0.05$). Lenalidomide caused a reduction in total cell numbers compared to the control at each time point and after 72 hrs was significantly lower than the control ($p<0.05$). Viability of HS5 cells exposed to lenalidomide was consistently above 75% at each time point.

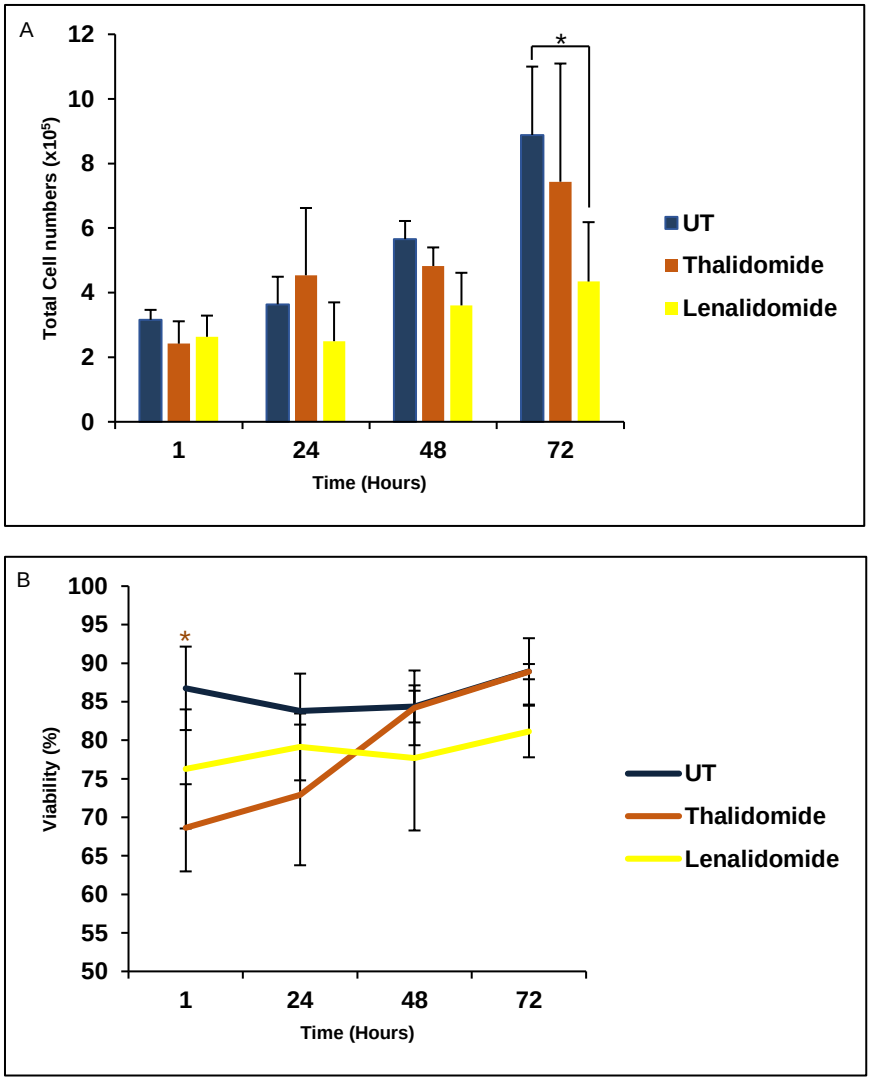


Figure 4.11 Total cell numbers (A) and viability (B) of HS5 cells cultured alone and after exposure to immunomodulatory agents. HS5 cells were cultured alone and exposed to either thalidomide (200 ng / ml) or lenalidomide (4 µM) for 1 hr. Cell numbers and viability were lower than the untreated (UT) 1 hr after treatment with thalidomide or lenalidomide however these improved at subsequent time points. Cell counts were measured at 1, 24, 48 and 72 hrs post exposure. Bars represent mean ± SD Significant differences between cells are indicated with an * (* $p<0.05$, ** $p<0.01$) Orange * when compared to thalidomide (n=3).

4.3.1.2.2 U266B1 cultured alone and treated with thalidomide or lenalidomide

Both thalidomide and lenalidomide treatment of U266B1 cells cultured alone resulted in total cell numbers similar to those unexposed to drug (figure 4.12). Viability of thalidomide exposed cells was consistent with that of the untreated with an improved viability at 72 hrs. Lenalidomide caused a reduction in cell viability at 24 hrs which then plateaued for the remainder of the experiment and at 72 hrs was significantly lower than that of the untreated ($p<0.05$). However viability still remained above 70%. These findings indicate that both thalidomide and lenalidomide do not cause cytotoixic effects on U266B1 cells when adminsitered at the MM clinical dose in an independent culture which is converse to the effects seen in HS5 when MM is supposed to be the target of these agents.

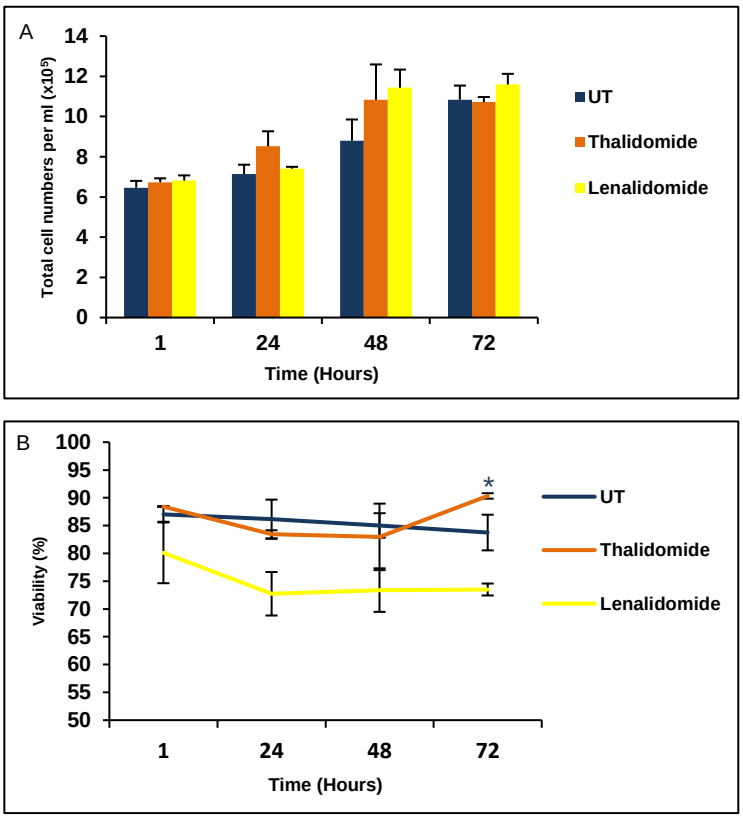


Figure 4.12 Total cell numbers (A) and viability (B) of U266B1 cells after exposure to immunomodulatory agents alone. U266B1 cells were cultured alone and exposed to either thalidomide (200 ng / ml) or lenalidomide (4 µM) for 1 hr. Thalidomide and lenalidomide did not produce cytotoxic effects against U266B1 cells when cultured independently compared to the untreated (UT). Cell counts were measured at 1, 24, 48 and 72 hrs post exposure. Bars represent a mean ± SD. Significant differences between cells are indicated with an *. Blue * when untreated is compared to lenalidomide (* $p<0.05$) (n=3).

4.3.1.2.3 HS5 cells co-cultured with U266B1 and exposed to thalidomide or lenalidomide

Treatment of HS5 cells with the immunomodulatory agents thalidomide and lenalidomide while in co-culture with U266B1 caused a reduction in total HS5 cell numbers at each time point (figure 4.13). Thalidomide treatment resulted in a significant decrease in total cell numbers at 72 hrs post exposure compared to the untreated ($p<0.05$). Lenalidomide treatment resulted in reduced numbers although this was not significant at any time point. However neither agent caused a significant effect on HS5 cell viability. Over the 72 hrs cell viability was consistently above 70% in all samples. These results suggest that MM cells have a detrimental effect on HS5 cells when in a non-contact co-culture.

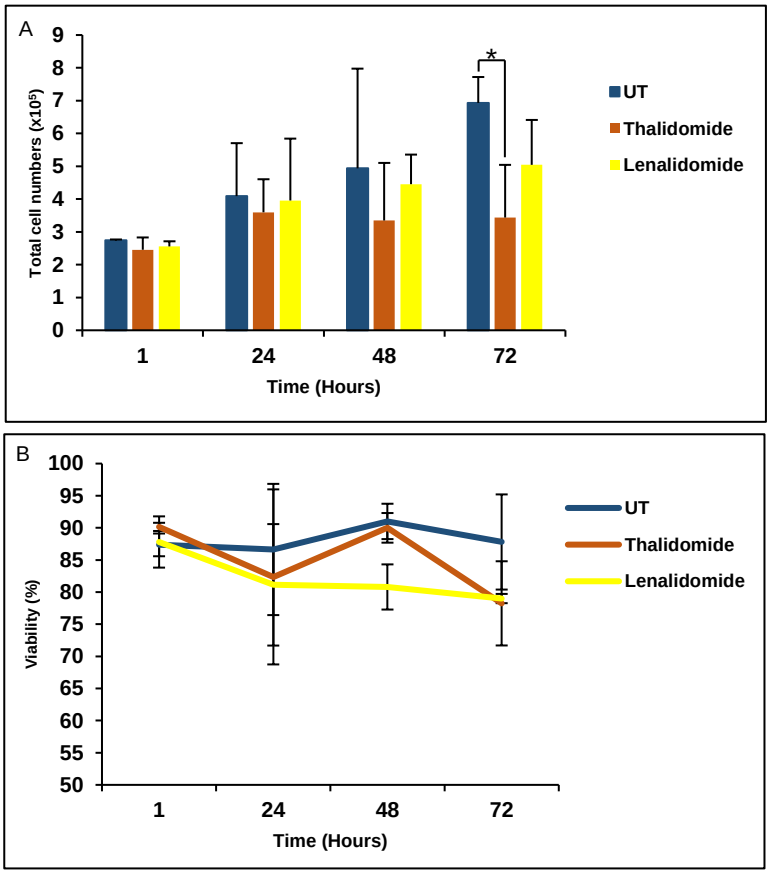


Figure 4.13 Total cell numbers (A) and viability (B) of HS5 cells after exposure to immunomodulatory agents while in co-culture with U266B1 cells. HS5 and U266B1 cells were cultured together via an insert and were exposed to either thalidomide (200 ng / ml) or lenalidomide (4 µM) for 1 hr. Total cell numbers were reduced following exposure to thalidomide over 72 hrs. Lenalidomide exposure also reduced total numbers of HS5 cells. Viability of HS5 cells exposed to either agent was similar to that of the untreated (UT). Cell counts were recorded at 1, 24, 48 and 72 hrs post exposure. Bars represent mean ± SD. Significant differences between cells are indicated with an * (* $p<0.05$) (n=3).

4.3.1.2.4 U266B1 cells co-cultured with HS5 cells and exposed to thalidomide or lenalidomide

Thalidomide and lenalidomide did not significantly affect total cell numbers of U266B1 cells in co-culture with HS5 (figure 4.14). Total cell numbers of U266B1 exposed to each immunomodulatory agent were higher than the untreated at 48 and 72 hrs post exposure.

Thalidomide did not cause significant effects on the viability of U266B1 cells when in co-culture with HS5 at any time point. Lenalidomide treatment resulted in a significant decrease in viability at 24 hrs ($p<0.05$) which gradually continued to decline at 48 hrs and was significantly lower at 72 hrs ($p<0.05$) although cell viability was still above 80%. Although these agents failed to produce significant cytotoxic effects on U266B1 cells when cultured alone, these findings indicate the protective effects of HS5 on U266B1 cells. However HS5 cells in co-culture had reduced cell numbers and appear to have in-fluxed the majority of the toxic effects (figure 4.13).

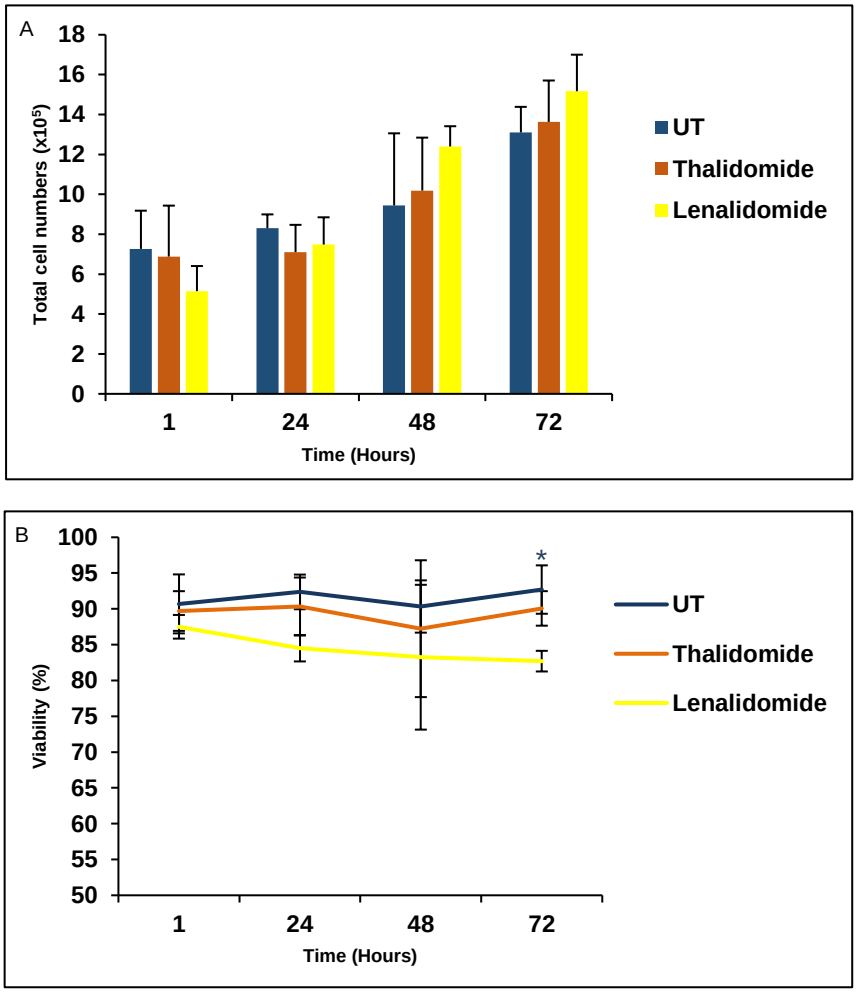


Figure 4.14 Total cell numbers (A) and viability (B) of U266B1 cells after exposure to immunomodulatory agents when in co-culture with HS5 cells. HS5 and U266B1 cells were cultured together via an insert and both cells exposed to either thalidomide (200 ng / ml) or lenalidomide (4 µM) for 1 hr. Immunomodulatory agents thalidomide and lenalidomide did not produce significant cytotoxic effects on U266B1 cells when in in co-culture with HS5 compared to the untreated (UT). Cell counts were measured at 1, 24, 48 and 72 hrs post exposure. Bars represent a mean ± SD. Significant differences between cells are indicated with an *. Blue * when compared to lenalidomide (* $p<0.05$) (n=3).

4.3.1.2.5 HS5 bystander model

To investigate whether a bystander effect occurs between these two cell types, HS5 and U266B1 were cultured separately and treated with either thalidomide or lenalidomide before being co-cultured with previously untreated U266B1 or HS5 cells respectively. HS5 cells that were treated with thalidomide and then co-cultured with untreated U266B1 cells had significantly lower total cell numbers at 72 hrs compared to those that were untreated and co-cultured with previously exposed U266B1 ($p<0.01$) (figure 4.15). Lenalidomide produced similar effects to thalidomide with total cell numbers lower than those that were not exposed to drug directly. However this was not significant at any time point. Viability remained high in those that were untreated and exposed to previously treated U266B1 (both thalidomide and lenalidomide). However, those that were initially treated with thalidomide had a decreased viability over 24 – 72 hrs, although this was not significant and viability remained above 70% at each time point.

These results suggest that HS5 cells are not protected by U266B1 cells when they are treated with either thalidomide or lenalidomide. A bystander effect from previously treated U266B1 cells did not appear to occur.

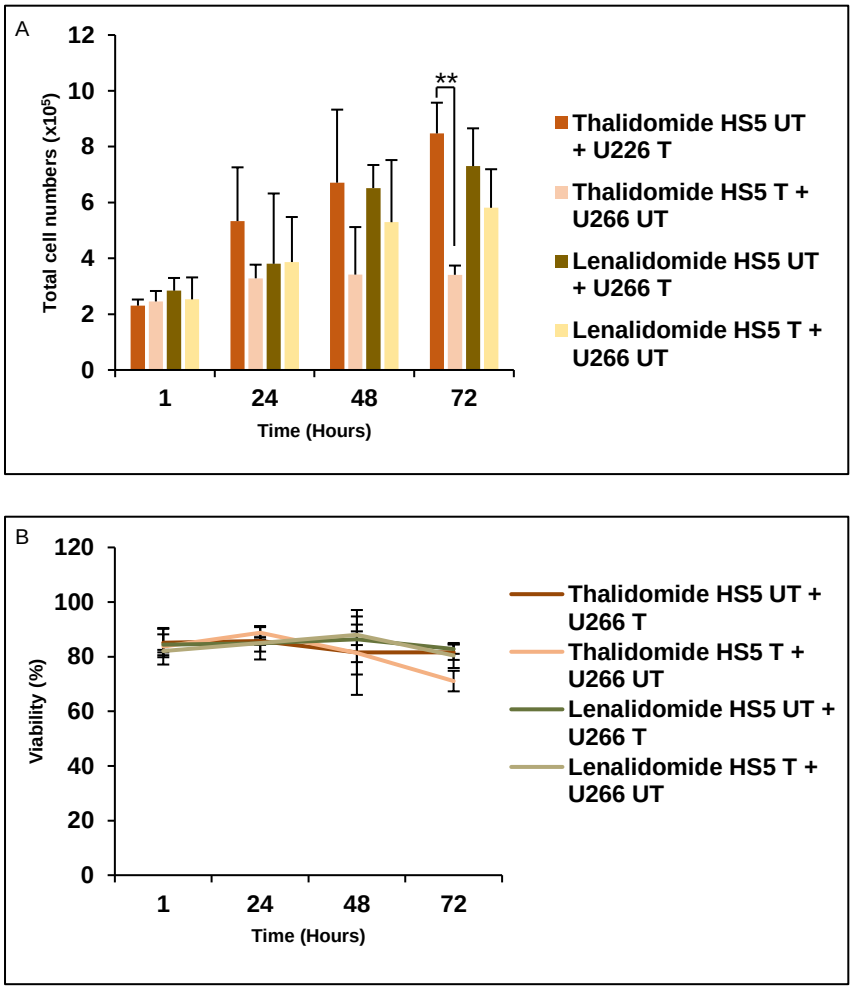


Figure 4.15 Total cell numbers (A) and viability (B) of HS5 cells after exposure to immunomodulatory agents either directly or indirectly via culture with treated U266B1 cells. HS5 were cultured alone and exposed to either thalidomide (200 ng / ml), lenalidomide (4 µM) for 1 hr or left untreated. These cells were then co-cultured (via an insert) with either untreated (UT), thalidomide (200 ng / ml) or lenalidomide (4 µM) treated U266B1. No bystander effect was observed between cells that were previously exposed to drug and cultured with untreated cells. Thalidomide treated HS5 had a reduction in HS5 cells numbers compared to the control at each time point. Cell counts were measured at 1, 16, 48 and 72 hrs post exposure. Abbreviations: HS5 UT + U266B1 T: HS5 cells untreated and co-cultured with treated U266; HS5 T + U266B1 UT: HS5 cells treated and co-cultured with untreated U266B1. Bars represent a mean ± SD . Significant differences between cells are indicated with an * (** $p<0.01$) (n=3).

4.3.1.2.6 U266B1 bystander model

U266B1 cells that were treated with thalidomide and then co-cultured with untreated HS5 did not show significant differences in total cell numbers compared to those that were untreated and co-cultured with previously exposed HS5 cells (figure 4.16). U266B1 cells that were untreated and co-cultured with HS5 previously exposed to lenalidomide had a significant improvement in total cell numbers at 48 hrs ($p<0.01$). U266B1 cells that were treated with lenalidomide showed a significant decrease in total cell numbers at 48 ($p<0.05$) and 72 hrs ($p<0.05$) compared to those that were untreated and co-cultured with previously exposed HS5. However those U266B1 cells that were directly exposed to lenalidomide and cultured with untreated HS5 had similar cell numbers to when cultured alone (figure 4.12). Viability of U266B1 cells exposed to each immunomodulatory agent, either directly or indirectly via the exposure to HS5 cells, was not significantly affected at any time point. These observations appear to indicate that neither of these agents significantly effect the survival of U266B1 cells when exposed to drug directly or indirectly via HS5, although HS5 cells in co-culture with U266B1 seem to promote the proliferation and promote the viability of U266B1 cells.

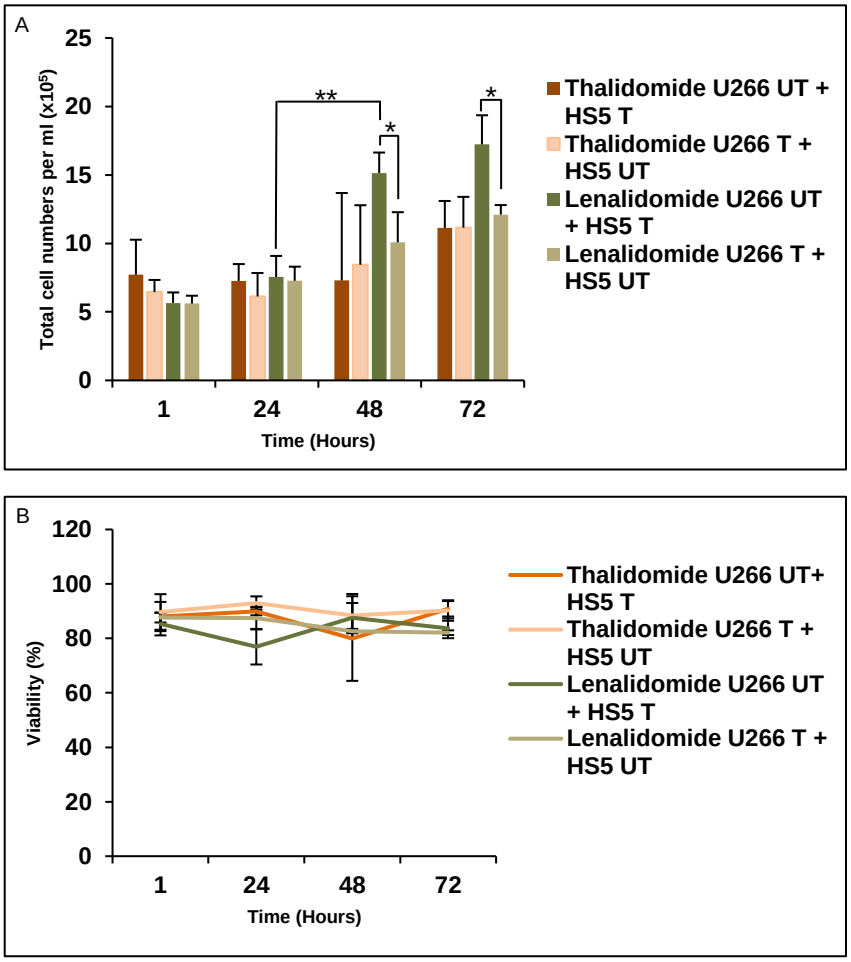


Figure 4.16 Total cell numbers (A) and viability (B) of U266B1 cells after exposure to immunomodulatory agents either directly or indirectly via culture with treated HS5 cells. U266B1 were cultured alone and exposed to either thalidomide (200 ng / ml) or lenalidomide (4 µM) for 1 hr or left untreated. These cells were then co-cultured (via an insert) with either untreated or thalidomide (200 ng / ml) or lenalidomide (4 µM) treated HS5 cells. No bystander effect was evident between cells that were previously exposed to drug and cultured with untreated cells**.** Cell counts were recorded at 1, 24, 48 and 72 hrs post exposure. Abbreviations: U266B1 UT + HS5 T: U266B1 cells treated and co-cultured with untreated HS5; U266B1 T + HS5 UT: U266B1 cells treated and co-cultured with untreated HS5. Bars represent a mean ± SD. Significant differences between cells are indicated with an * (*p<0.05, **p<0.01) (n=3).

4.3.1.3 Proteasome inhibitors

HS5 stromal cells and U266B1 cells were exposed to either bortezomib (500 nM) or carfilzomib (13.8 nM) for 1 hr either alone or in co-culture (via an insert). Samples were taken immediately (1 hr), 24, 48 and 72 hrs after exposure.

4.3.1.3.1 HS5 cells cultured alone and exposed to bortezomib or carfilzomib

Proteasome inhibitors, bortezomib and carfilzomib, had significant cytotoxic effects against HS5 cells when cultured alone (figure 4.17). There was a significant decline in total cell numbers 24 hrs post exposure to bortezomib compared to the control ($p<0.05$). HS5 cells numbers continued to decrease at 48 ($p<0.01$) and at 72 hrs ($p<0.001$) post exposure to bortezomib compared to the control. Viability of HS5 cells exposed to bortezomib also declined at each time point and was significantly lower than the control at 48 ($p<0.001$) and 72 hrs ($p<0.001$) post exposure. Carfilzomib treatment resulted in a decrease in cell numbers at each time point that was significantly lower than the control at 48 ($p<0.001$) and 72 hrs ($p<0.001$) post exposure. Viability was also significantly lower than the control at 24 ($p<0.05$), 48 ($p<0.01$) and 72 hrs ($p<0.01$) compared to untreated HS5 cells.

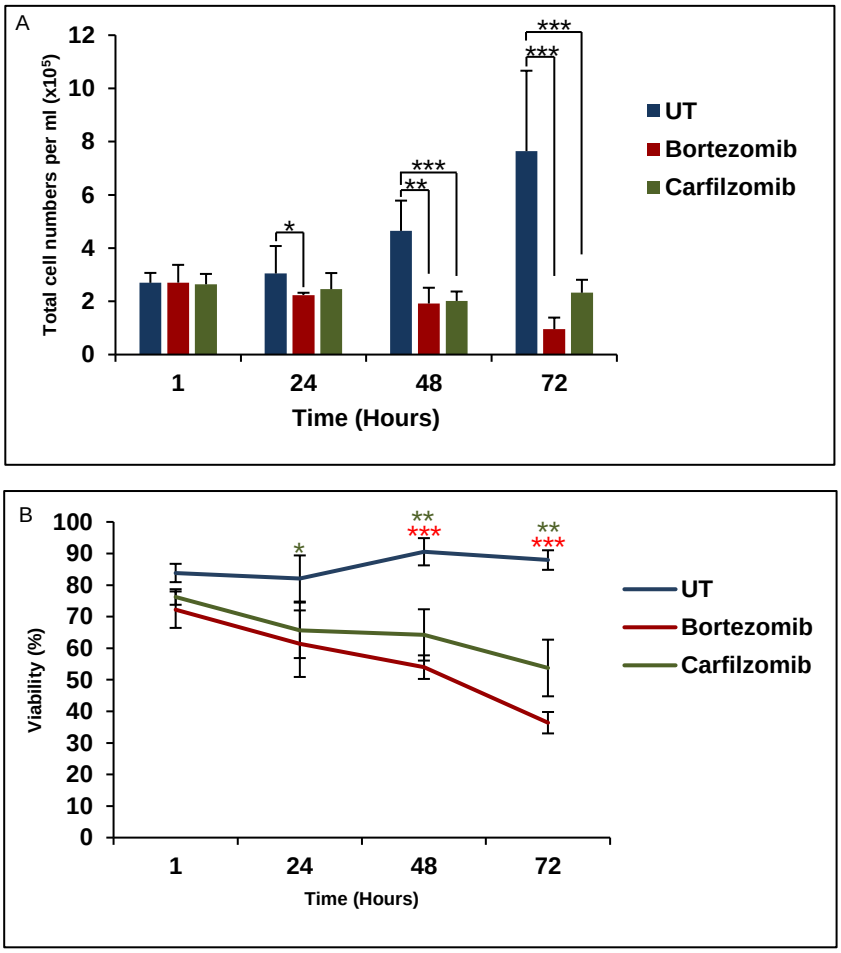


Figure 4.17 Total cell numbers (A) and viability (B) of HS5 cells after exposure to proteasome inhibitors when cultured alone. HS5 cells cultured alone were exposed to either bortezomib (500 nM) or carfilzomib (13.8 nM) for 1 hr. Both bortezomib and carfilzomib exposure resulted in significant cytotoxic effects against HS5 cells compared to the untreated (UT). Cell counts were recorded at 1, 24, 48 and 72 hrs post exposure. Bars represent a mean ± SD. Significant differences between cells are indicated with an * Red * when compared to bortezomib, green * when compared to carfilzomib (* $p<0.05$, ** $p<0.01$, ** $p<0.001$) (n=3).

4.3.1.3.2 U266B1 cultured alone and exposed to bortezomib or carfilzomib

U266B1 cells cultured alone and exposed to bortezomib for 1 hr had a significant reduction in the total numbers of cells at 24 hrs post exposure ($p<0.001$) which continued to be significantly lower than the control at all subsequent time points (48 hrs $p<0.01$, 72 hrs $p<0.001$) (figure 4.18). Carfilzomib treatment did not have a significant detrimental effect on the total cell numbers until 48 hrs post exposure ($p<0.001$) which remained significant at 72 hrs post exposure ($p<0.001$). Viability was severely affected as a result of exposure to each of these agents. Viability decreased at each time point and at 48 and 72 hrs post exposure to both carfilzomib and bortezomib there was a significant decrease in U266B1 cell viability ($p<0.001$).

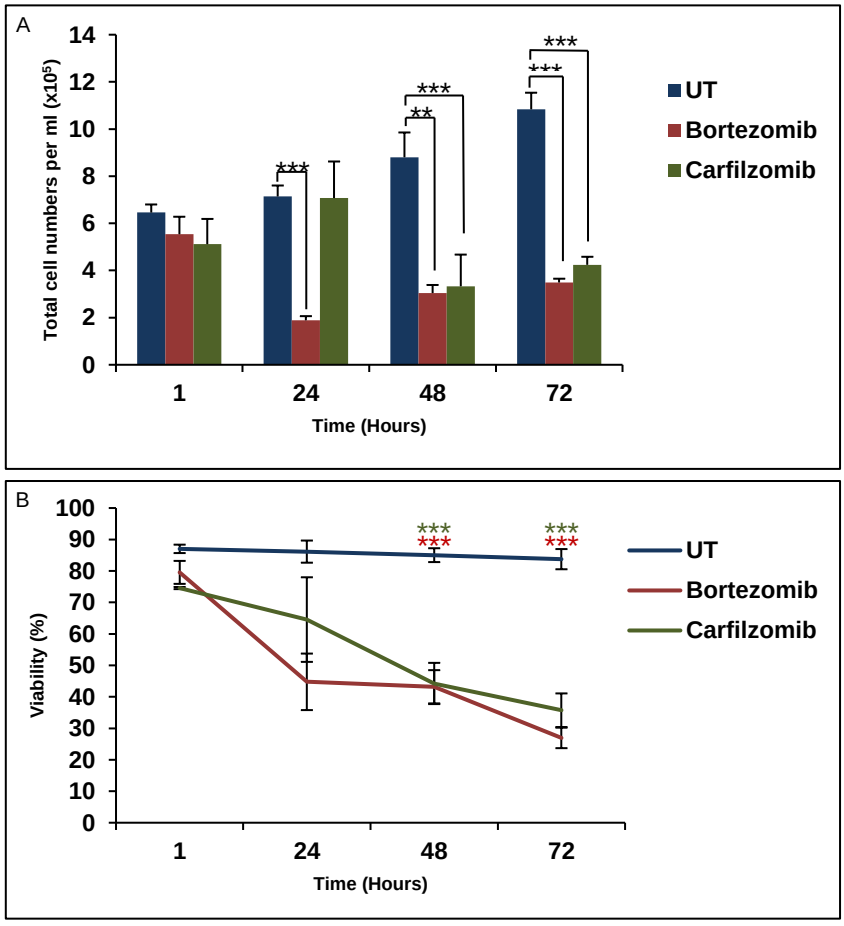


Figure 4.18 Total cell numbers (A) and viability (B) of U266B1 cells after exposure to proteasome inhibitors when cultured alone. U266B1 cells were cultured alone and exposed to either bortezomib (500 nM) or carfilzomib (13.8 nM) for 1 hr. Both agents caused a reduction in the total cell numbers and viability of U266B1 cells compared to the untreated (UT). Cell counts were measured at 1, 24, 48 and 72 hrs post exposure. Bars represent a mean ± SD.Significant differences between cells are indicated with an *. Red * when compared to bortezomib, green * when compared to carfilzomib (** $p<0.01$, *** $p<0.001$) (n=3).

4.3.1.3.3 HS5 cells co-cultured with U266B1 cells and exposed to bortezomib or carfilzomib

When HS5 cells were exposed to bortezomib in a co-culture with U266B1, there was a reduction in the numbers of HS5 cells and their viability (figure 4.19). Immediately after exposure to bortezomib there were significantly lower total cell numbers of U266B1 cells compared to the untreated ($p<0.05$) which improved slightly at 24 hrs. However, cell numbers continued to decrease at 48 ($p<0.001$) and 72 hrs ($p<0.01$), compared to the control. Cell viability following bortezomib treatment significantly decreased at 24 hrs ($p<0.01$) and continued to significantly decrease at 48 ($p<0.05$) and 72 hrs ($p<0.001$) compared to the control.

However, HS5 cells exposed to carfilzomib in a co-culture with U266B1 had improved total cell numbers compared to those exposed to bortezomib. Cell numbers were significantly lower than the control at 48 hrs ($p<0.05$) and were lower than the control at 72 hrs but not significantly. Cell viability decreased at each time point following exposure to carfilzomib and was significantly lower than the control at 24 ($p<0.01$) and 72 hrs ($p<0.001$).

These findings indicate that U266B1 cells exacerbate the cytotoxic effects of bortezomib on HS5 cells with viability further reduced when compared to HS5 cells cultured alone and exposed to drug (figure 4.17). Cell numbers following exposure to carfilzomib were improved compared to independent culture. However viability of these cells was simillar to that of alone culture.

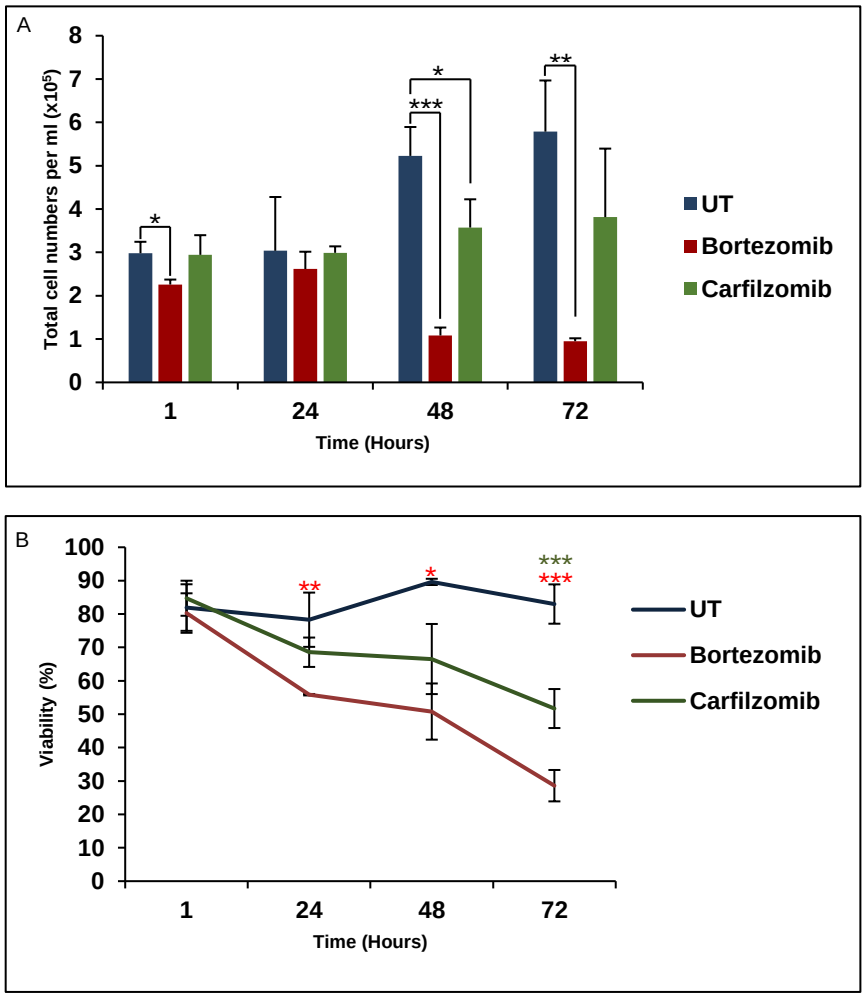


Figure 4.19 Total cell numbers (A) and viability (B) of HS5 cells after exposure to proteasome inhibitors in co-culture with U266B1 cells. HS5 cells co-cultured with U266B1 via an insert were exposed to either 500 nM bortezomib or 13.8 nM carfilzomib for 1 hr. Both proteasome inhibitors exposure resulted in significant cytotoxic effects against HS5=] cells when in co-culture with U266B1 compared to the untreated (UT). Cell counts were recorded at 1, 24, 48 and 72 hrs post exposure. Bars represent a mean ± SD. Significant differences between cells are indicated with an *. Red * when compared to bortezomib, green * when compared to carfilzomib (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) (n=3).

4.3.1.3.4 U266B1 cells co-cultured with HS5 cells and exposed to bortezomib or carfilzomib

HS5 cells provide protection from the cytotoxic effects of both bortezomib and carfilzomib on U266B1 cells (figure 4.20). Total cell numbers following exposure to bortezomib were significantly decreased at 24 hrs ($p<0.05$) and thereafter (48; $p<0.05$, 72; $p<0.01$). Viability after exposure to bortezomib continued to decline significantly at 24 ($p<0.05$) and 48 hrs ($p<0.01$) before reaching a plateau at 72 hrs. However viability of these cells was still significantly reduced at 72 hrs ($p<0.01$).

Carfilzomib exposure did not result in the drop in total cell numbers that was seen when exposed to drug alone although these cells were still significantly lower in number at 24 ($p<0.05$) and 72 hrs ($p<0.05$) compared to the control. U266B1 cells exposed to carfilzomib had a consistent viability over 48 hrs that was not significantly different from the control, before decreasing at 72 hrs ($p<0.05$).

It is important to note that cell viability did not decrease to as low a percentage as that seen when U266B1 cells were cultured alone. Furthermore, as HS5 cells had a decreased viability when in co-culture with U266B1 (figure 4.19) and with U266B1 cells having an improved viability, these results suggest that HS5 cells provide protection to U266B1 cells at a consequence to their own viability. Moreover, untreated U266B1 cells also had increased cell numbers when in co-culture with HS5 compared to independent cultures.

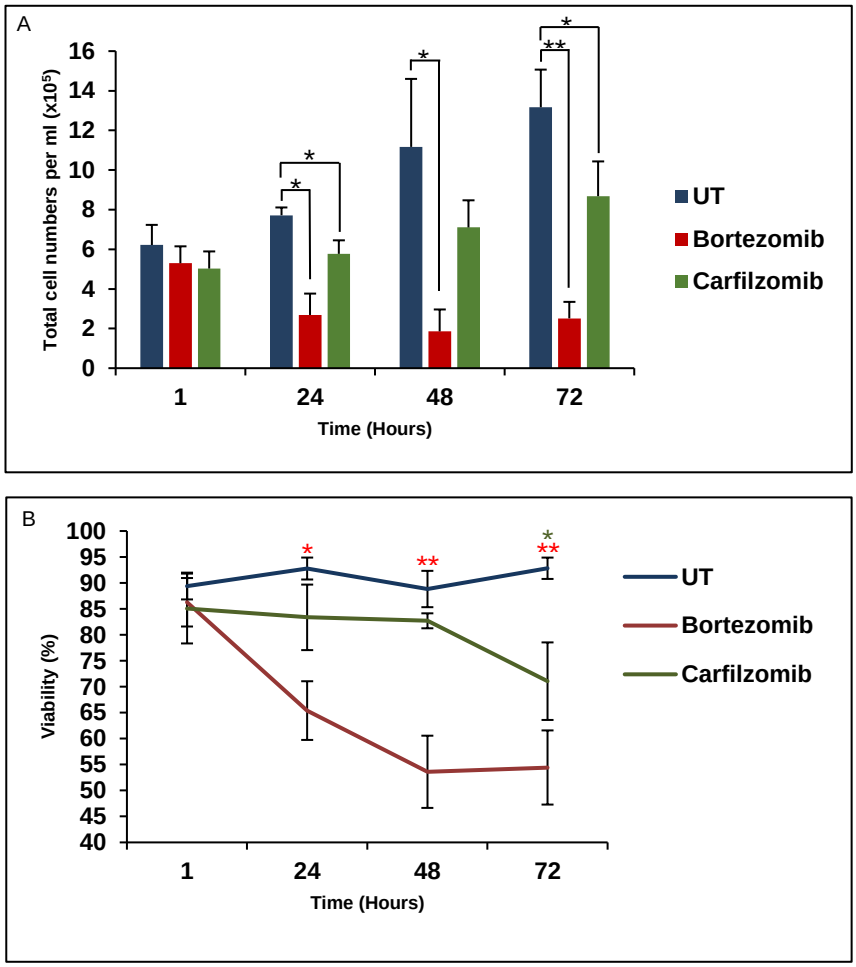


Figure 4.20 Total cell numbers (A) and viability (B) of U266B1 cells after exposure to proteasome inhibitors when in co-culture with HS5 cells. HS5 and U266B1 cells were co-cultured via an insert and exposed to either 500 nM bortezomib or 13.8 nM carfilzomib for 1 hr. Total numbers of U266B1 cells decreased following exposure to proteasome inhibitors in co-culture with HS5 cells compared to the untreated (UT). Cell viability decreased following exposure to bortezomib before reaching a plateau at 72 hrs. Carfilzomib treatment did not produce as significant an effect as bortezomib although viability of U266B1 cells decreased at 72 hrs. U266B1 cell counts were measured at 1, 24, 48 and 72 hrs post exposure. Bars represent a mean ± SD. Significant differences between cells are indicated with an *. Red * when compared to bortezomib, green * when compared to carfilzomib (* $p<0.05$, ** $p<0.01$) (n=3).

4.3.1.3.5 HS5 bystander model

To investigate whether a bystander effect occurs between these two cell types following exposure to proteasome inhibitors, HS5 and U266B1 cells were cultured separately and treated with either bortezomib or carfilzomib before being co-cultured with previously untreated U266B1 or HS5 cells respectively. Bortezomib exposed HS5 cells that were cultured with untreated U266B1 had similar numbers to HS5 cells that were untreated and cultured with previously exposed U266B1 cells at 1 and 16 hrs (figure 4.21). At 48 hrs HS5 cells that were previously exposed to bortezomib had significantly reduced cell numbers compared to those that were untreated ($p<0.01$) with further reductions at 72 hrs ($p<0.01$). Viability of HS5 cells that were exposed to bortezomib was also significantly reduced at 24 ($p<0.05$), 48 ($p<0.01$) and 72 hrs post exposure ($p<0.05$). HS5 cell numbers that were not directly exposed to drug and cultured with previously treated U266B1 increased at each time point and had a viability consistently above 80%.

HS5 that were not exposed to carfilzomib directly did not have a significant reduction in total cell numbers compared to HS5 cells that were treated and cultured with untreated U266B1 cells (figure 4.21). HS5 cells that were treated with carfilzomib and then co-cultured with untreated U266B1 cells had lower total cell numbers than those not exposed to carfilzomib directly, at each time point with a significant reduction at 72 hrs ($p<0.05$). Viability of these previously carfilzomib exposed HS5 cells was also reduced at each time point with a significant reduction at 72 hrs ($p<0.001$). Untreated HS5 cells' viability that were cultured with U266B1 cells that were previously exposed to carfilzomib was unaffected. These findings suggest that a bystander effect has not taken place in HS5 cells with results similar to those presented earlier in HS5 cells cultured alone and directly exposed to each of the proteasome inhibitors (figure 4.17). Furthermore these results show that U266B1 cells do not protect HS5 from the cytotoxic effects of these agents but rather contribute to an increase in damage to these cells.

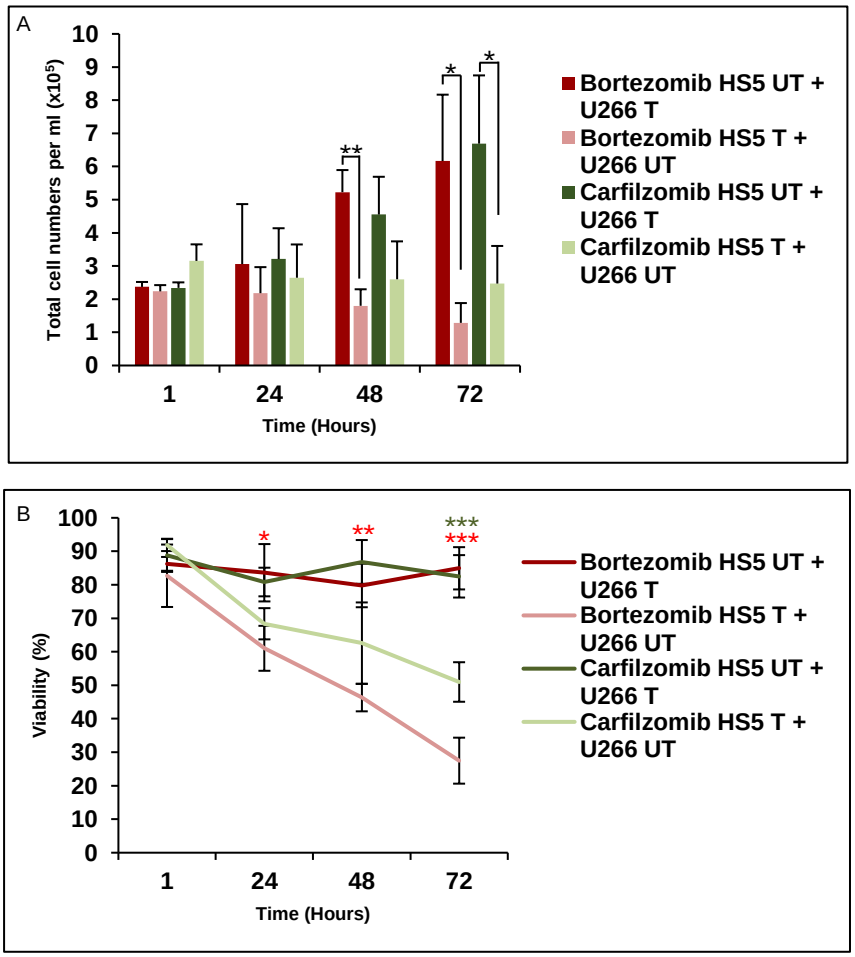


Figure 4.21 Total cell numbers (A) and viability (B) of HS5 cells after exposure to proteasome inhibitors either directly or when co-cultured with previously exposed U266B1 cells. HS5 cells were cultured alone and exposed to either 500 nM bortezomib or 13.8 nM carfilzomib for 1 hr or left untreated. These cells were then co-cultured (via an insert) with either untreated or bortezomib / carfilzomib treated U266B1. Cells not directly exposed to proteasome inhibitors did not experience a significant decrease in cell numbers or viability at any time point. HS5 cells directly exposed to each of the proteasome inhibitors had reduced cell numbers and viability at each time point compared to those cells that were not directly exposed to drug. Cell counts were recorded at 1, 24, 48 and 72 hrs post exposure. Abbreviations: HS5 UT + U266B1 T: HS5 cells untreated and co-cultured with treated U266B1; HS5 T + U266B1 UT: HS5 cells treated and co-cultured with untreated U266B1. Bars represent a mean ± SD. Significant differences between cells are indicated with an *. Red * when compared to bortezomib, green * when compared to carfilzomib (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) (n=3).

4.3.1.3.6 U266B1 bystander model

Both U266B1 cells that were treated directly with bortezomib and those cultured with previously bortezomib treated HS5 cells, had a reduction in their total cell numbers over 72 hrs (figure 4.22). U266B1 cells that were untreated and cultured with previously bortezomib treated HS5 did not increase in number at any time point. However cell numbers remained higher than those that were directly exposed to bortezomib. Viability of these untreated cells remained above 85% at each time point. U266B1 cells that were directly exposed to bortezomib and cultured with untreated HS5 had decreased cell numbers at 24 ($p<0.05$) and 48 hrs ($p<0.05$) compared to those that were not exposed to drug directly. Cell numbers then improved at 72 hrs and were not significantly different to those that were not exposed to drug directly ($p>0.05$). Viability decreased at each time point, and was significantly lower at 72 hrs ($p<0.01$). With viability below 40% when exposed to bortezomib in independent culture (figure 4.18) these results suggest HS5 cells have provided some protection from bortezomib's cytotoxic effects.

U266B1 cells that were treated with carfilzomib and then co-cultured with untreated HS5 did not show significant differences in total cell numbers compared to those U266B1 that were untreated and co-cultured with previously treated HS5. Viability of U266B1 cells in those exposed to carfilzomib either directly or indirectly via an insert was not significantly affected at any time point. With viability significantly reduced when exposed to carfilzomib when cultured alone (figure 4.18), these observations suggest again that HS5 cells have provided some protection to U266B1 cells from the cytotoxic effects seen when exposed to drug alone.

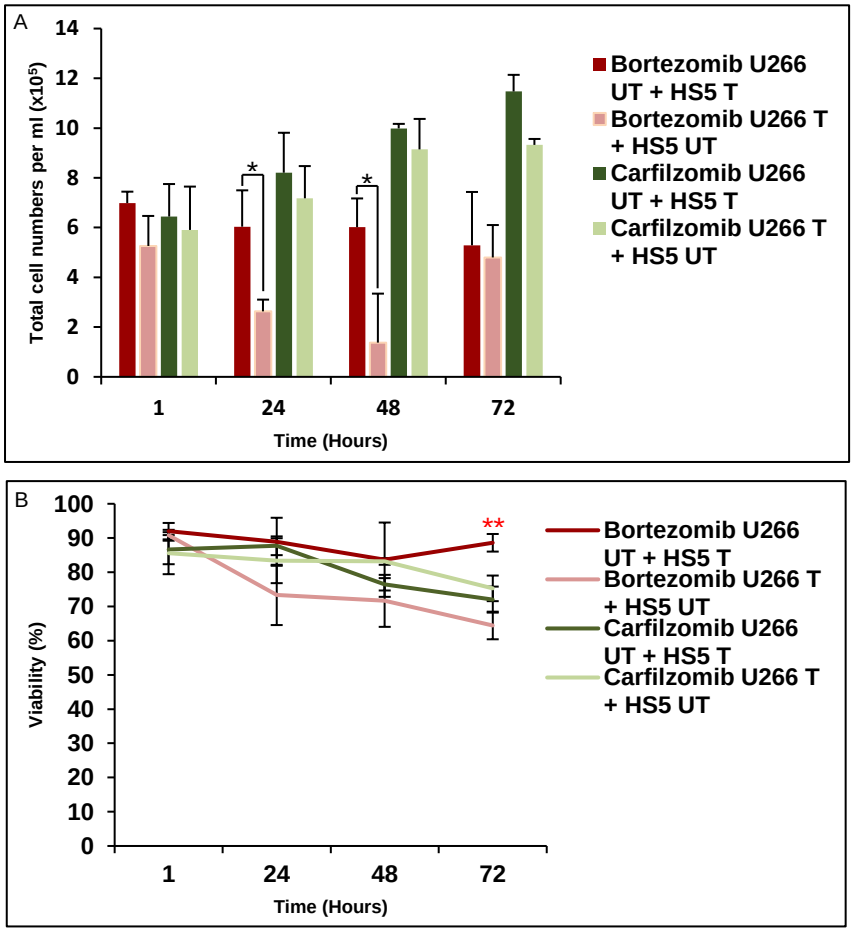


Figure 4.22 Total cell numbers (A) and viability (B) of U266B1 cells after exposure to proteasome inhibitors either directly or when co-cultured with previously exposed HS5 cells. U266B1 cells were cultured alone and exposed to either bortezomib (500 nM) or carfilzomib (13.8 nM) for 1 hr or left untreated. These cells were then co-cultured (via an insert) with either untreated or bortezomib / carfilzomib treated HS5. Total cell numbers of U266B1 cells are not significantly affected 72 hrs post exposure to proteasome inhibitors either directly or indirectly when in co-culture with HS5 cells. Viability of U266B1 cells is reduced following exposure to bortezomib directly. Viability of U266B1 exposed directly to carfilzomib is not significantly different to U266B1 cells left untreated and co-cultured with previously treated HS5 cells. Abbreviations: U266B1 UT + HS5 T: U266B1 cells treated and co-cultured with untreated HS5; U266B1 T + HS5 UT: U266B1 cells treated and co-cultured with untreated HS5. Cell counts were recorded at 1, 24, 48 and 72 hrs post exposure. Bars represent a mean ± SD. Significant differences between cells are indicated with an *. Red * when compared to bortezomib (* $p<0.05$, ** $p<0.01$) (n=3).

4.3.2 Phase contrast microscopy

4.3.2.1 HS5 morphology when cultured independently

HS5 stromal cells have distinct structural properties from those of fully differentiated cells (Choi *et al*., 2014, Roecklein and Storb, 1995). In standard 2D culture, HS5 stromal cells are maintained in an undifferentiated state and have a fibroblast-like appearance. Here HS5 stromal cells were cultured in complete DMEM/F12 medium and exposed to each of the chemotherapeutic agents described previously. The morphology of these cells was monitored by phase contrast microscopy at baseline (prior to chemotherapy), 16 hrs (for melphalan) / 24 hrs (for every other drug except melphalan), 48 and 72 hrs post exposure. Their morphology was visualized when cultured alone or in the presence of U266B1 cells (separated by an insert). Following the exposure of the HS5 cell line cultures to the chemotherapeutic agents, in an independent culture, structural abnormalities were seen (figures 4.23 – 4.25).

HS5 cells that were cultured alone in a 12 well plate and were not exposed to a chemotherapeutic agent exhibited a uniform fibroblast-like appearance and reached a confluent layer by 72 hrs (figures 4.23 - 4.25). At each time point these cells had a small cell body with small projections emanating from their centre. HS5 cells were found to lose their typical morphology following exposure to certain chemotherapeutic agents. Cells that were exposed to melphalan for 1 hr had a morphology similar to that of the untreated, although their cell bodies appeared more elongated than the control at 72 hrs. Melphalan exposed cells were also not as confluent as those that were untreated, which was evident at each time point (figure 4.23).

Thalidomide appeared to have no effect on the morphology of the HS5 cells and these also reached a confluent layer at 72 hrs (figure 4.24). Cells that were exposed to lenalidomide however, appeared to have large spaces between neighbouring cells at 24 and 48 hrs with cells having stellate processes. There was also evidence of lymphoblast-like cells (cells are spherical in shape and are in suspension) at each time point with cells

aggregated at 72 hrs and also failing to reach confluence by this time point (figure 4.24).

HS5 cells that were exposed to the proteasome inhibitors bortezomib and carfilzomib suffered the most significant changes to their morphology giving rise to a highly disorganised arrangement of cells at 24, 48 and 72 hrs (figure 4.25). It can be clearly seen that cells treated with bortezomib had an altered morphology with cells becoming lymphoblast-like and detaching from the surface of the plate. Furthermore, many cells have begun to clump together at 24 hrs. Cells appear to have regained some structural normality at 48 hrs. However their morphology has become exacerbated further at 72 hrs with cells becoming aggregated once more (figure 4.25). Carfilzomib treatment also caused an alteration to the morphology of HS5 cells giving rise to aggregated and lymphoblast-like cells that had detached from the surface of the plate at 24, 48 and 72 hrs (figure 4.25).

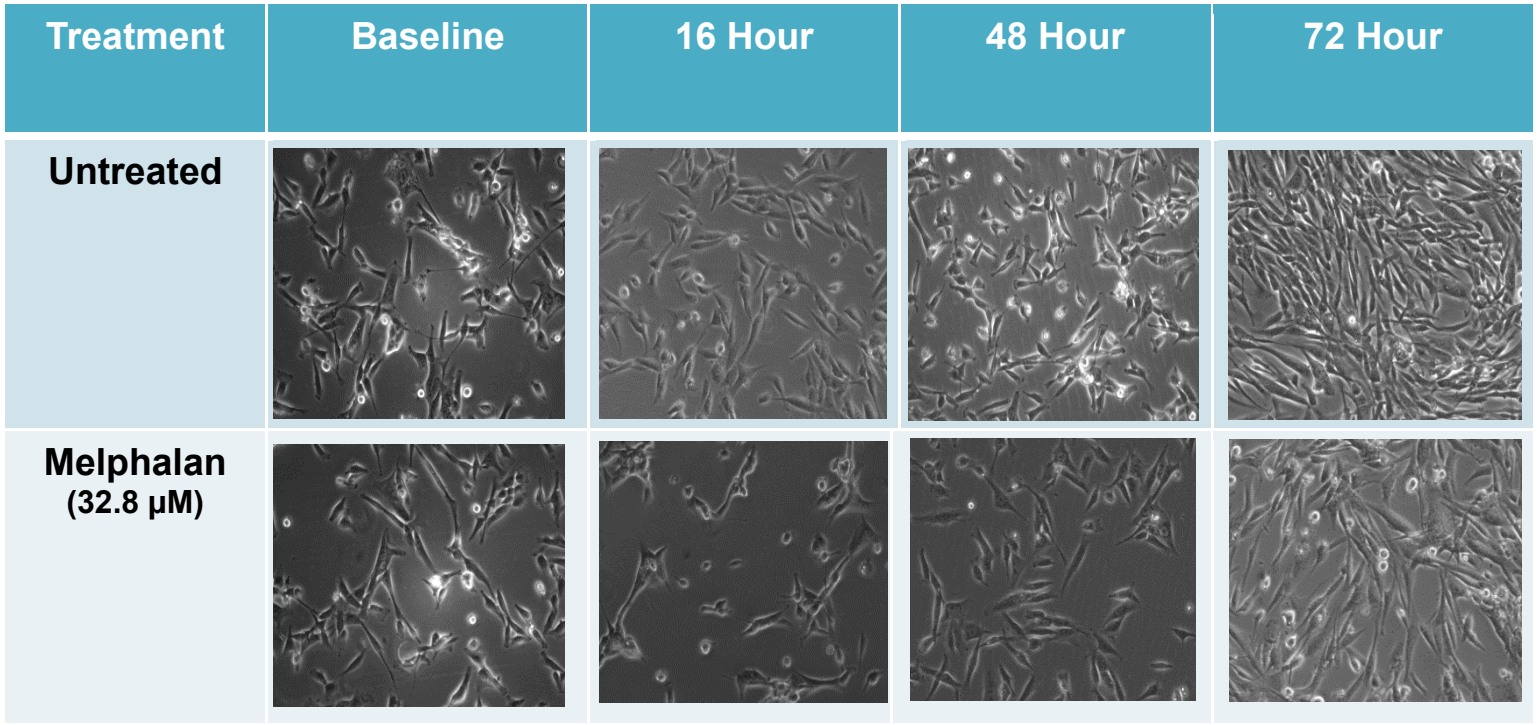


Figure 4.23 Representative images of HS5 morphology following 1 hr exposure to melphalan at the clinically relevant dose compared to an untreated control. Melphalan (32.8 µM) exposed cells are loosely aggregated at 24 hrs with improved morphology at 48 and 72 hrs. However they fail to reach the same confluence as the untreated cells at 72 hrs. Images were taken at baseline (prior to treatment) and at 16, 48 and 72 hrs (post treatment). All images are x 10 magnification (representative of n=3).

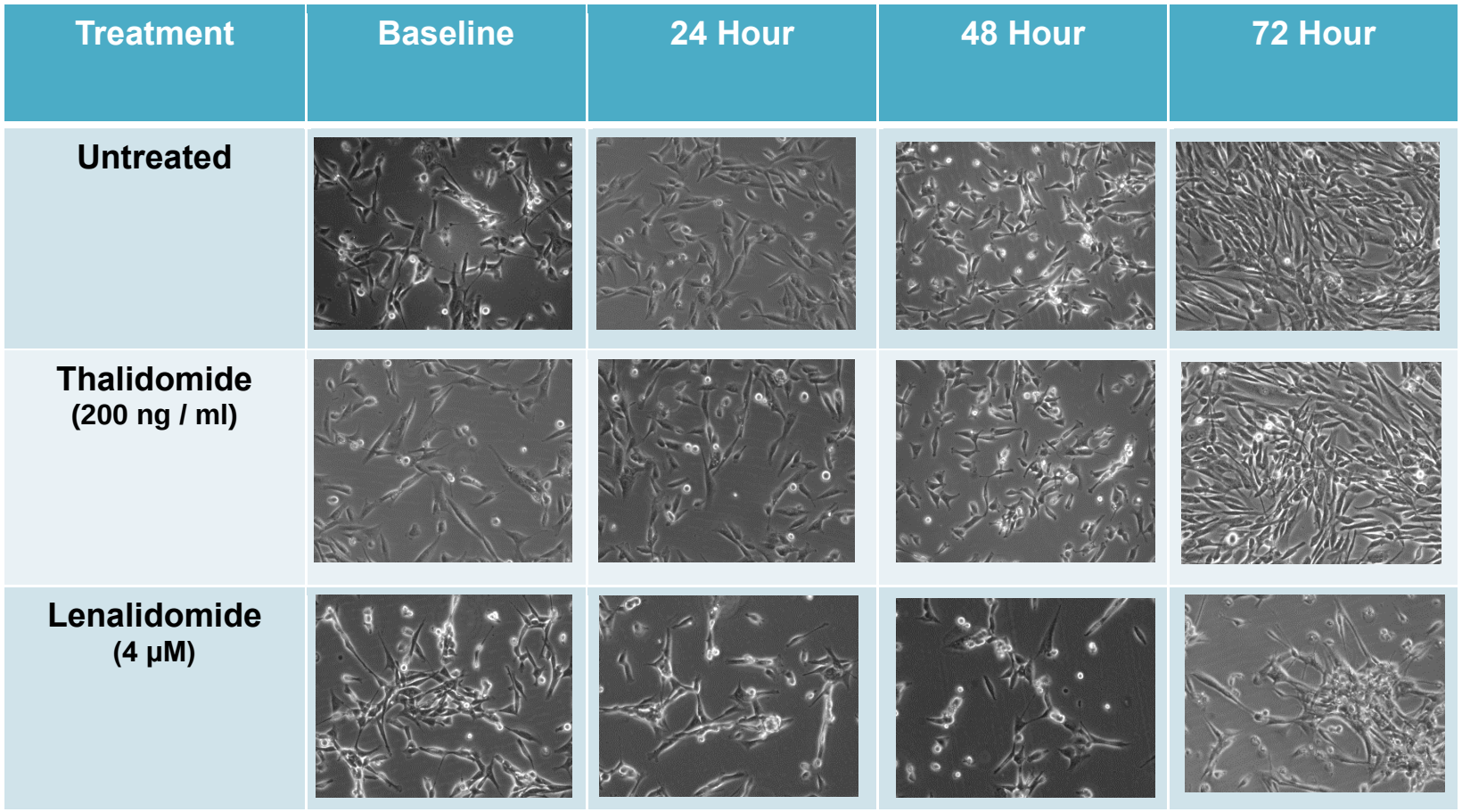


Figure 4.24 Representative images of HS5 morphology following 1 hr exposure with immunomodulatory agents at the clinically relevant dose compared to an untreated control. Thalidomide (200 ng / ml) did not appear to have any significant effects on the morphology of HS5 when cultured alone. Lenalidomide (4 µM) exposed HS5 were fewer in number with increased aggregation of cells with stellate processes. Images were taken at baseline (prior to treatment) and at 24, 48 and 72 hrs (post treatment). All images are x 10 magnification (representative of n=3).

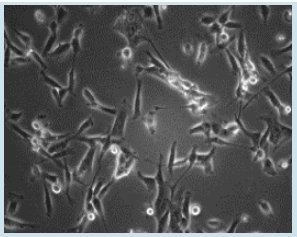
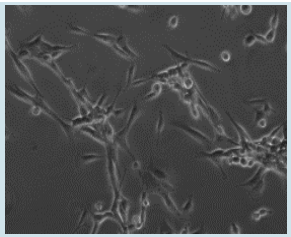
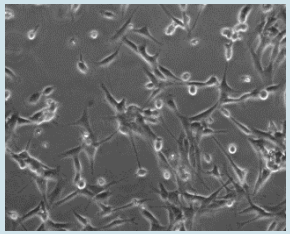
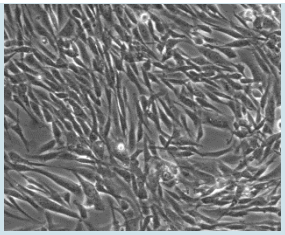
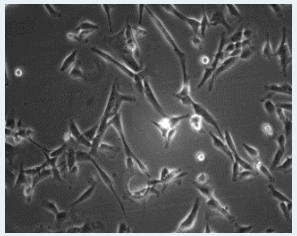
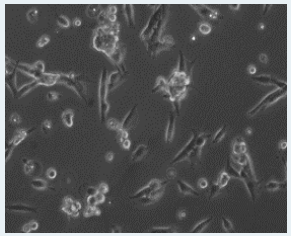
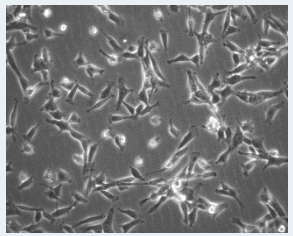
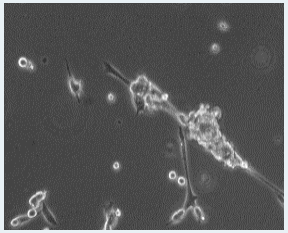
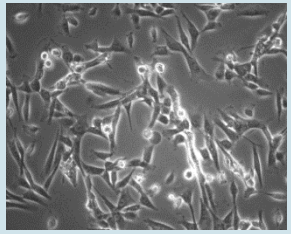
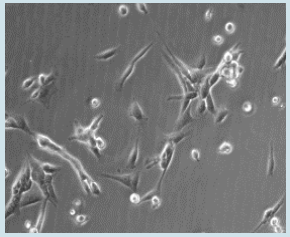
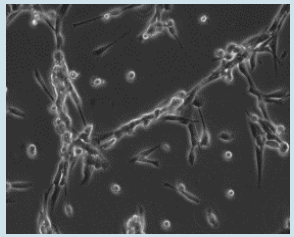
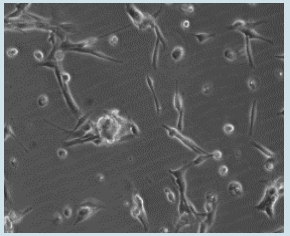
Treatment	Baseline	24 Hour	48 Hour	72 Hour
Untreated				
Bortezomib (500 nM)				
Carfilzomib (13.8 nM)				

Figure 4.25 Representative images of HS5 morphology following 1 hr exposure with proteasome inhibitors compared to an untreated control. HS5 become loosely aggregated and lymphoblast-like (rounded, detached from the surface of the plate) following exposure to 500 nM bortezomib and 13.8 nM carfilzomib. Images were taken at baseline (prior to treatment) and at 24, 48 and 72 hrs (post treatment). All images are x 10 magnification (representative of n=3).

4.3.2.2 HS5 morphology when co-cultured with U266B1 cells

When observing the unexposed HS5 cells in a co-culture with U266B1 cells, it was noted that they had a reduced proliferation and a mixed morphology compared to those that were cultured alone, the latter of which grew as a typical densely packed fibroblast-like monolayer (figures 4.26 – 4.28). The morphological changes that were seen for some agents when HS5 cells were cultured alone, were further exacerbated when in co-culture with U266B1. In each experiment both HS5 and U266B1 cells were exposed to chemotherapy for 1 hr while in co-culture.

When HS5 cells and U266B1 cells were exposed to melphalan in a co-culture there was a clear reduction in numbers and also a change in HS5 cellular morphology compared to the untreated cells. At 48 hrs cell bodies have become elongated with cells beginning to 'clump' together. Cells were fully aggregated at 72 hrs forming a large mass, with an increase in lymphoblast-like cells visible (figure 4.26).

The immunomodulatory agents thalidomide and lenalidomide both caused detrimental effects to the morphology of the HS5 cells (figure 4.27). Cells exposed to thalidomide saw an increase in lymphoblast-like cells at 24 and 48 hrs compared to the untreated. At 72 hrs cell morphology was similar to that of the untreated cells but there was a clear reduction in cell numbers and cells failed to reach a confluent layer. HS5 morphology following lenalidomide treatment resembled that of the untreated cells at 24 and 48 hrs. However at 72 hrs, cells had become aggregated and developed stellate processes while it can also been seen that some cells had become lymphoblast-like and detached from the plate (figure 4.27).

Upon treatment with the proteasome inhibitors bortezomib and carfilzomib, significant abnormalities were noted at each time point in the HS5 co-cultures (figure 4.28). There was an increase in lymphoblast-like cells at 24 hrs following treatment with bortezomib with cells being spherical in shape and in suspension. This increased after 48 hrs with further detachment from the plate at 72 hrs. Cells treated with carfilzomib displayed a highly diverse

pattern of growth. At 24 hrs there were large spaces between neighbouring HS5 cells with a large proportion of cells becoming lymphoblast-like and detached from the plate at 48 and 72 hrs. Furthermore, there was a clear aggregation of cells at 48 and 72 hrs with HS5 cell bodies having stellate processes.

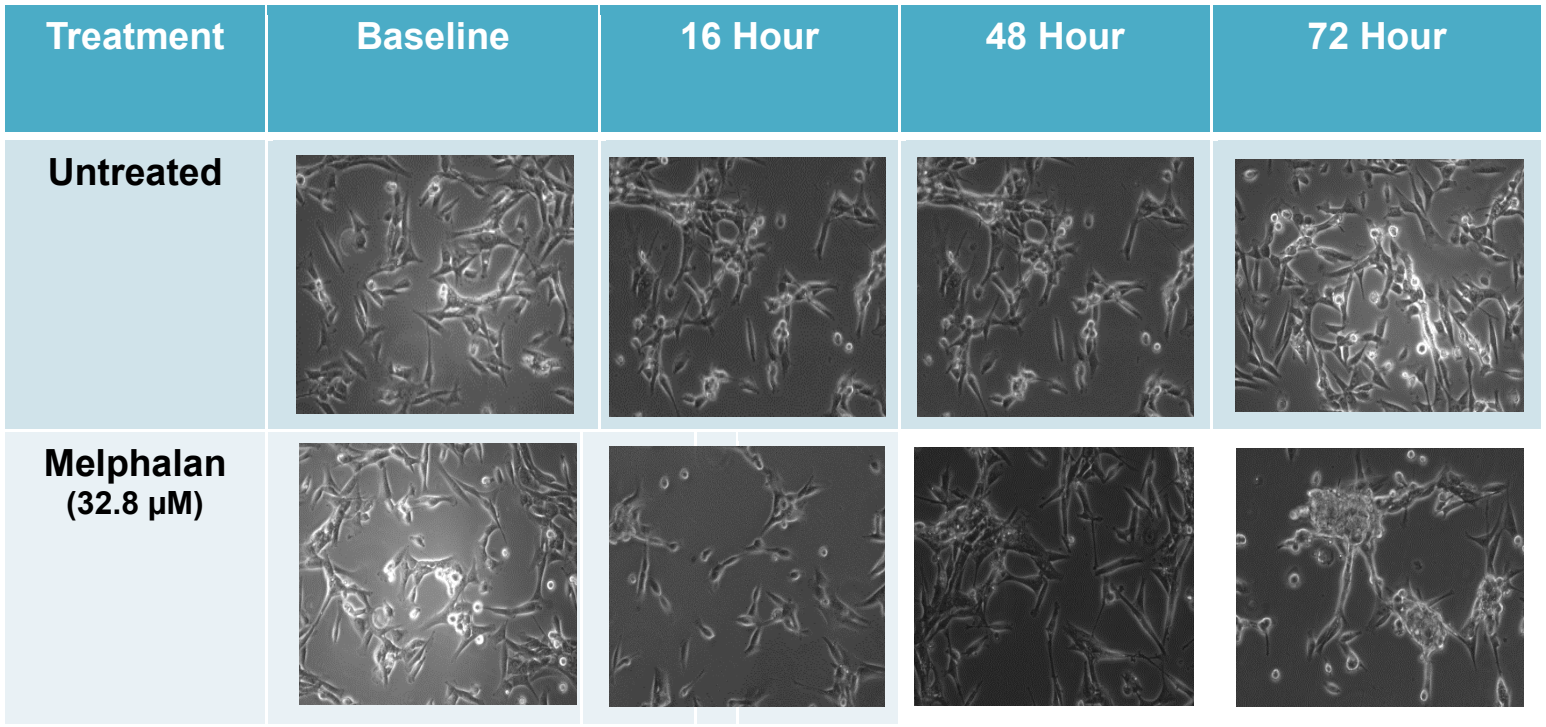


Figure 4.26 Phase contrast images of HS5 cells following co-culture with U266B1 with and without treatment with melphalan at the biologically relevant dose for 1 hr. Morphology of HS5 is affected when in co-culture with U266B1 cells evidenced by elongated cell bodies, an increase in rounded cells in suspension and also a failure to reach a confluent layer. This is further exacerbated by melphalan (32.8 µM) treatment which is profoundly evident at 72 hrs post exposure where there is an aggregation of cells with stellate processes. Images were taken at baseline (prior to treatment) and at 16, 48 and 72 hrs (post treatment). All images are x 10 magnification (representative of n=3).

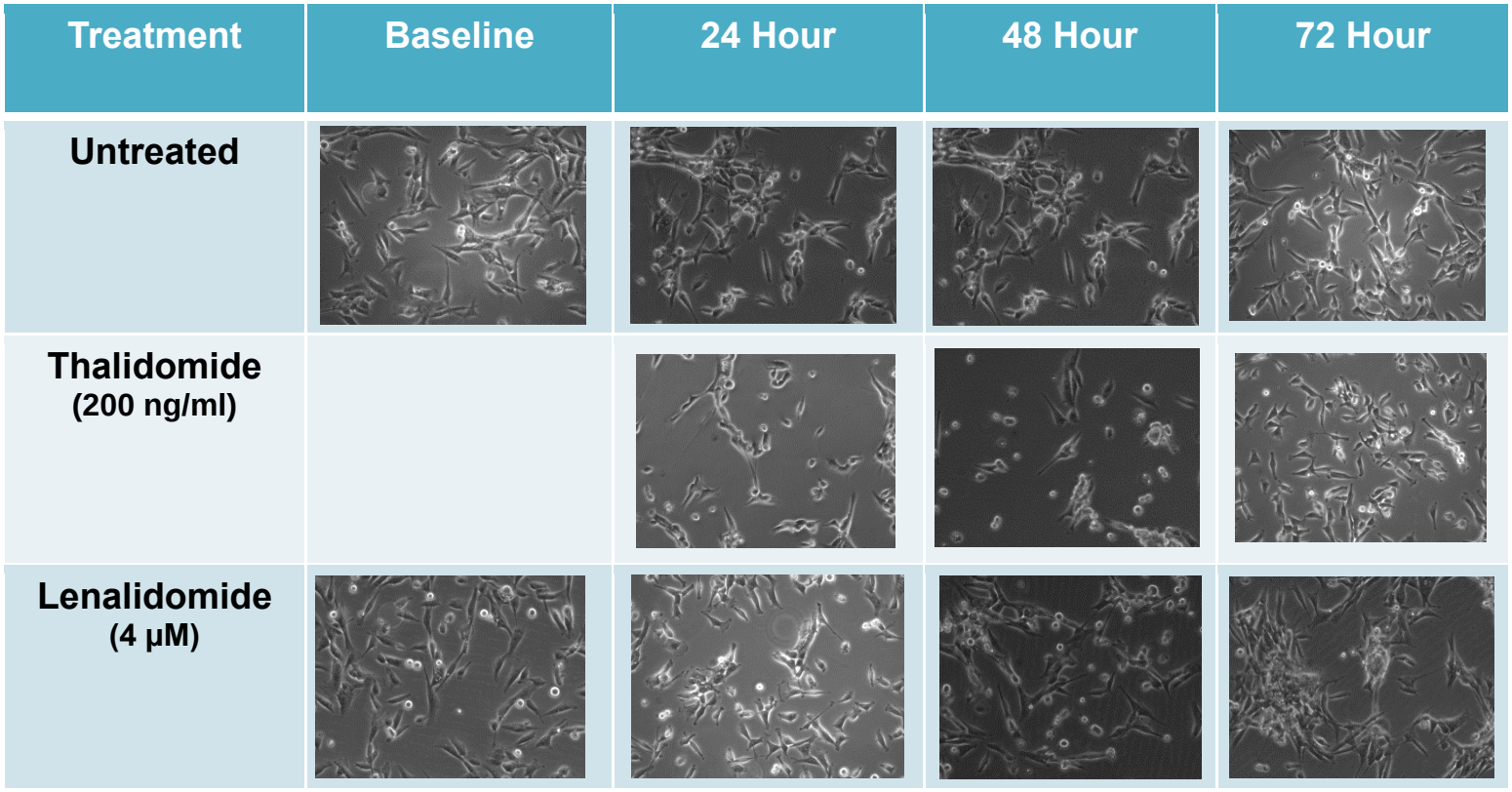


Figure 4.27 Phase contrast images of HS5 cells following co-culture with U266B1 with and without treatment with immunomodulatory agents at clinically relevant doses for 1 hr. HS5 morphology is affected by immunomodulatory agents thalidomide (200 ng / ml) and lenalidomide (4 µM) when administered in co-culture with U266B1. Both agents caused an increase in rounded cells in suspension with a reduced cell density. Lenalidomide treatment also resulted in cells becoming aggregated at 72 hrs forming distinct large masses. Images were taken at baseline (prior to treatment) and at 24, 48 and 72 hrs (post treatment). All images are x 10 magnification (representative of n=3).

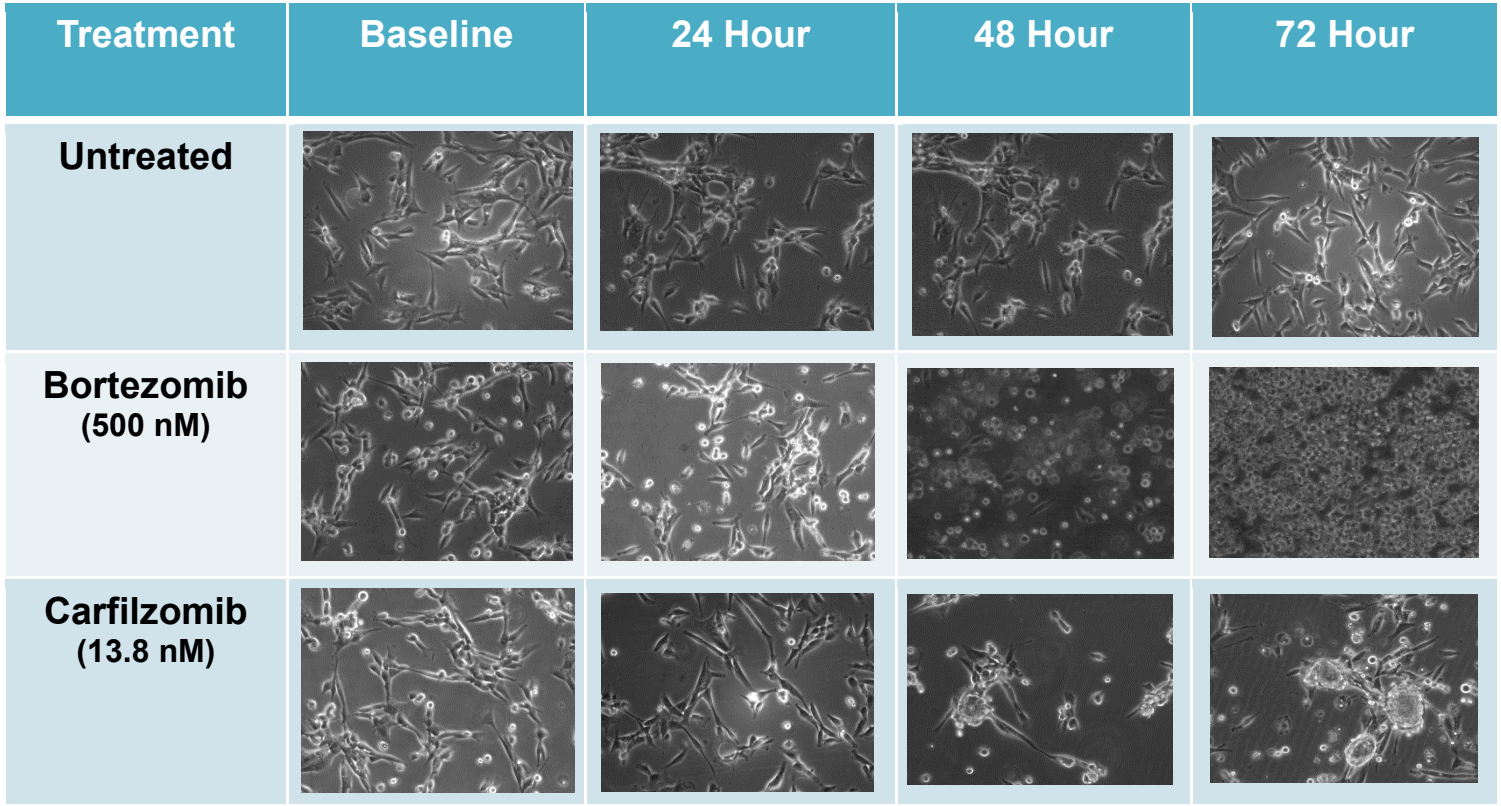


Figure 4.28 Phase contrast images of HS5 cells following co-culture with U266B1 with and without treatment with proteasome inhibitors at biologically relevant concentrations for 1 hr. Bortezomib (500 nM) exposed HS5 in co-culture with U266B1 become rounded in shape and are detached from the plate at 48 hrs which is further exacerbated at 72 hrs. Carfilzomib (13.8 nM) caused HS5 cells to aggregate at 48 hrs post exposure and this effect was intensified at 72 hrs. Images were taken at baseline (prior to treatment) and at 24, 48 and 72 hrs (post treatment). All images are x 10 magnification (representative of n=3).

4.3.3 HS5 Cell Differentiation

The ability of MSCs to undergo osteogenic and adipogenic differentiation in the presence of appropriate environmental stimuli is well established *in vitro*; however the ability of the HS5 stromal cell line to differentiate into these lineages is less well documented. Furthermore the ability of MSC and HS5 to differentiate in an *in vitro* model of MM is unknown. The differentiation potential of HS5 cells was investigated in cells cultured alone and in the presence of U266B1 cells (separated by an insert). HS5 cells were also stimulated to differentiate into osteogenic and adipogenic lineages following exposure to clinical doses of melphalan, bortezomib, carfilzomib, thalidomide and lenalidomide either alone or in the presence of U266B1 cells.

4.3.3.1 Osteogenic differentiation

To confirm osteogenic differentiation of the HS5 cells, cytochemical staining for the bone marker, alkaline phosphatase (ALP) was performed. HS5 cells were either cultured alone or with U266B1 cells separated by a transwell insert. Phase contrast images of differentiated HS5 cells clearly revealed that differentiated cells have an altered morphology. After 10 days of culture in differentiation medium, HS5 cells that were untreated changed from a fibroblastic-like appearance to a more cuboidal shape with small extensions from their cell body. These cells produced a large confluent layer of osteoblasts expressing high levels of ALP as evidenced by a dark blue appearance following staining with SIGMA FAST BCIP/NBT substrate when visualised microscopically (figure 4.29A). It can be seen from figure 4.29 that the ability of HS5 cells to differentiate when cultured alone can be affected by the exposure to chemotherapeutic agents.

HS5 previously exposed to melphalan are noticeably the most affected with the morphology of the HS5 cells completely altered with a severe enlargement of the cell body (figure 4.29B). However blue staining evidencing ALP activity is visible. Treatment with the immunomodulatory agents thalidomide and lenalidomide does not appear to have caused significant hindrance to the cells' ability to differentiate. A uniform layer of

cells was formed with blue staining visible comparable to the untreated sample (figure 4.29 C and D). Carfilzomib treated cells also did not show significant disruption to osteogenic differentiation, with a confluent layer of HS5 cells visible with their cell bodies staining blue, evidencing ALP activity (figure 4.29E). However it can be seen that those cells previously exposed to bortezomib have become lymphoblast-like and are detached from the surface of the plate. Blue staining evidencing ALP activity was visible although a confluent layer of cells failed to form (figure 4.29F).

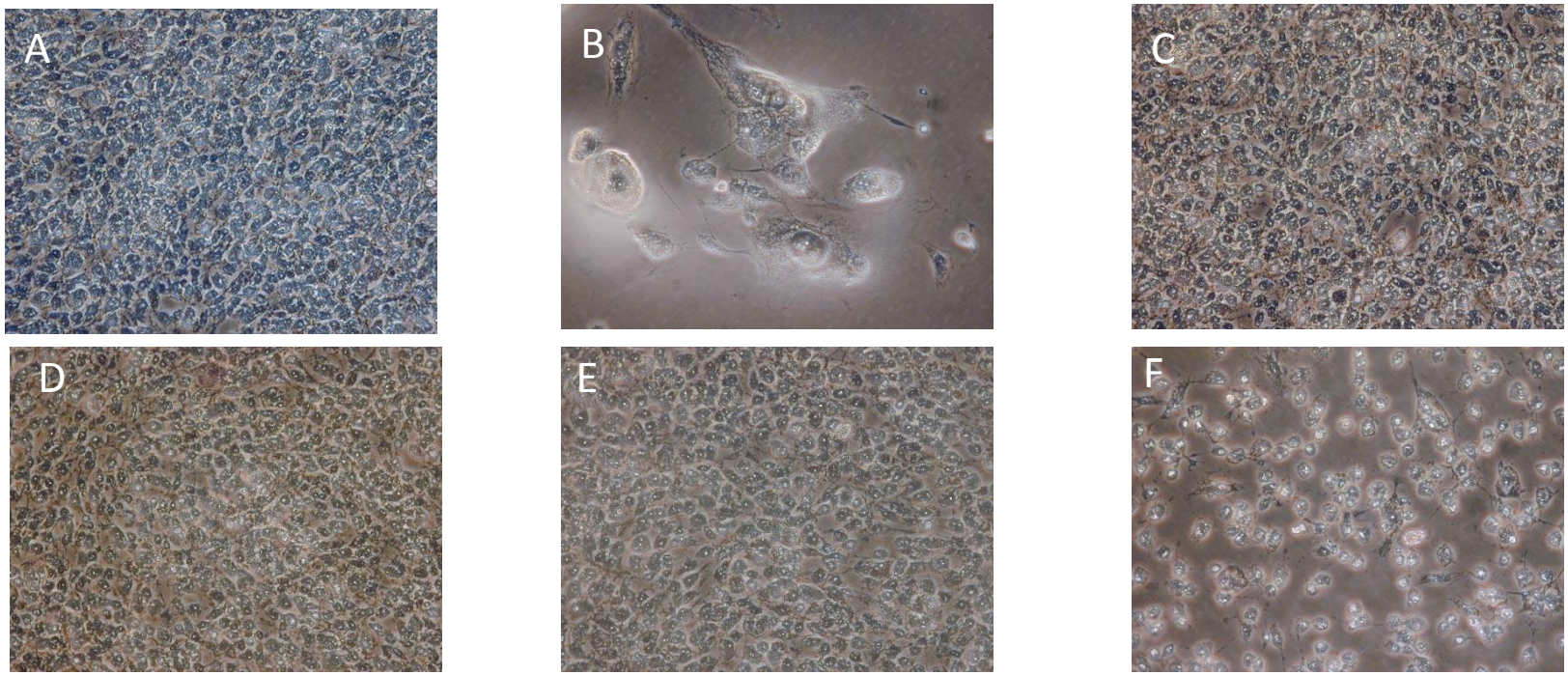


Figure 4.29 Representative images of HS5 cells cultured independently and differentiated along osteogenic lineages following chemotherapy exposure. HS5 were seeded at 3 x 10^4 cells per well, in 1.5 mls Osteodiff medium and were maintained with complete replacement of medium every 3 or 4 days. A – HS5 cells untreated, B – Melphalan (32.8 µM), C – Thalidomide (200 ng / ml), D – Lenalidomide (4 µM), E – Carfilzomib (13.8 nM), F – Bortezomib (500 nM). Osteogenic differentiation of HS5 cells was evidenced by the blue staining of cells with SIGMA FAST BCIP/NBT substrate indicating alkaline phosphatase activity. Osteogenic differentiation following melphalan treatment was severely disrupted (B). Bortezomib treatment drastically altered the morphology of HS5 cells with cells becoming rounded and lymphoblast like (F). Osteogenic images were taken at day 10. (B - x 40 magnification, all other images x 20 magnification, representative of n=3).

Osteogenic differentiation of HS5 cells in the presence of U266B1 cells was evident when left unexposed to chemotherapy with cells expressing ALP giving rise to their dark blue appearance (figure 4.30). These differentiated HS5 cells had a similar morphology to that when stimulated to differentiate alone, having a cuboidal appearance with small thin projections from their cell bodies. However there were significantly lower numbers of HS5 cells visible with large spaces between neighbouring cells. This finding correlates with the similar findings seen from previous proliferation and undifferentiated morphology results.

Osteogenic differentiation was also observed in HS5 cells previously exposed to melphalan in co-culture with U266B1. ALP activity evidenced by blue staining was visible, although the cell morphology was altered with cells having elongated cell bodies with stellate processes (figure 4.30B). However visible numbers of these cells were severely reduced. Thalidomide and lenalidomide treatment did not cause a significant alteration in HS5 ability to differentiate compared to the untreated (figure 4.30 C and D). The morphology of these cells was similar to that of the control with both having a cuboidal appearance. ALP activity was also evident with the cells expressing a blue appearance. Carfilzomib exposure did not alter the osteogenic differentiation capacity of these cells, giving rise to a differentiated cell type similar to that of the untreated (figure 4.30E). However treatment of HS5 cells with bortezomib in co-culture with U266B1 had a severe effect on the morphology and differentiation of these cells. HS5 cells became lymphoblast-like and dissociated from the surface of the well and resulting in loss from the well during staining and subsequently could not be shown here.

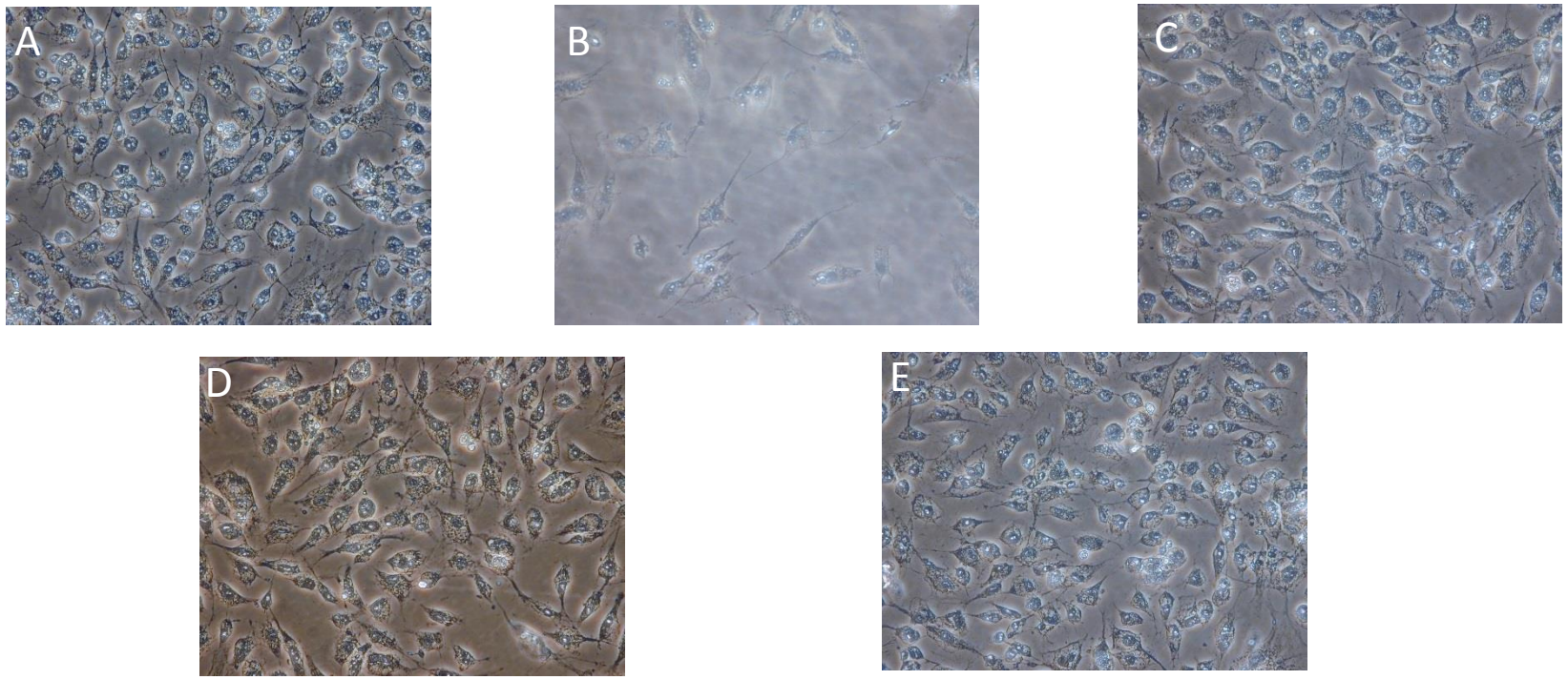


Figure 4.30 Representative images of HS5 cells co-cultured with U266B1 and differentiated along osteogenic lineage following chemotherapy exposure. HS5 were seeded at 3 x 10^4 cells per well, with 5 x 10^5 U266B1 cells in an insert, together in 1.5 mls Osteodiff medium and were maintained with complete replacement of medium every 3 or 4 days. A – HS5 cells untreated, B – Melphalan (32.8 µM), C – Thalidomide (200 ng / ml), D – Lenalidomide (4 µM), E – Carfilzomib (13.8 nM). Osteogenic differentiation in the presence of U266B1 cells was evidenced by blue staining of cells with SIGMA FAST BCIP/NBT substrate indicating alkaline phosphatase activity. However there were fewer cells compared to the independent cultures. Melphalan severely disrupted the morphology of differentiated HS5 cells. Osteogenic images were taken at day 10. (All images x 20 magnification, representative of n=3).

4.3.3.2 Adipogenic differentiation

To confirm adipogenic differentiation of the HS5 cells, cells were stained for lipid containing vacuoles with oil red O following either independent culture or in the presence of U266B1 cells separated by a transwell insert. After 21 days in AdipoDiff culture medium, HS5 cells cultured alone and unexposed to drug were successfully induced to differentiate down the adipogenic lineage visualised by the abundant accumulation of lipid-containing vacuoles which stained red with oil red O (figure 4.31A). The ability of HS5 cells to differentiate when cultured alone can be affected by exposure to chemotherapeutic agents.

HS5 cells that were exposed to melphalan as with the osteogenic differentiation, were much fewer in number, having a dramatic change in their morphology, with their cell bodies increasing in size and a failure to produce lipid containing vacuoles (figure 4.31B). No lipid containing vacuoles were observed in thalidomide treated HS5 cells although the morphology of these cells appeared unhindered (figure 4.31C). In contrast lenalidomide however, does not appear to have caused significant limitation to the cells' ability to differentiate into adipocytes, with a uniform layer of cells formed with lipid containing vacuoles present (figure 4.31D). HS5 cells exposed to carfilzomib had more lipid containing vacuoles compared to the proteasome inhibitor bortezomib. However, there were fewer adipocytes visible compared to the untreated (figure 4.31, E). Adipocyte differentiation of HS5 cells was severely affected following bortezomib treatment, with only a small number of lipid containing vacuoles observed (figure 4.31F).

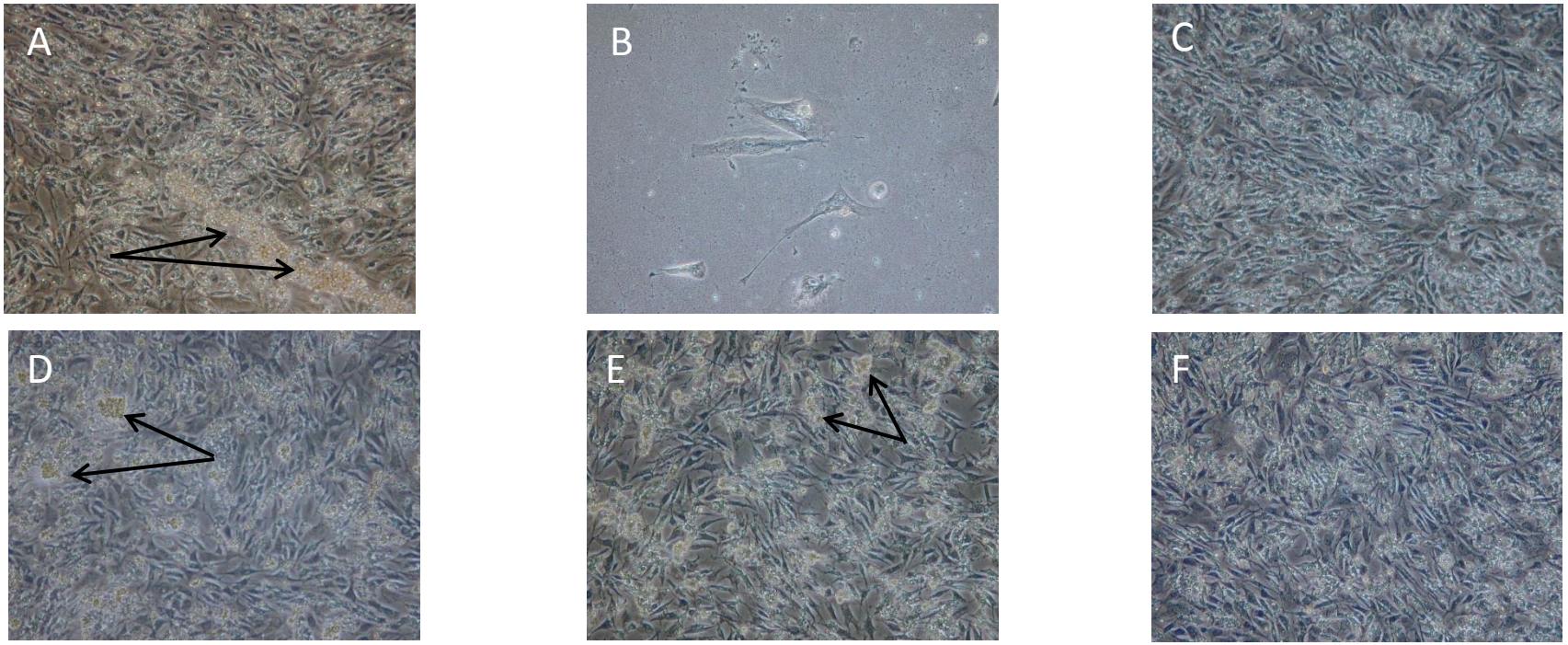


Figure 4.31 Images depicting HS5 cells cultured alone, differentiated along adipogenic lineages following chemotherapy exposure. HS5 were seeded at 5 x 10^4 per well in 1.5 mls adipogenic medium and were maintained by complete medium depletion every 3 or 4 days. Adipocytes were confirmed by Oil Red O staining on day 21 and images taken. A – HS5 cells untreated, B – Melphalan (32.8 µM), C – Thalidomide (200 ng / ml), D – Lenalidomide (4 µM), E – Carfilzomib (13.8 nM), F - Bortezomib (500 nM). Adipogenic differentiation was significantly reduced after exposure to melphalan as evidenced by the lack of lipid-containing vacuoles and their severely altered morphology. Adipogenic differentiation appeared reduced in all other chemotherapy treated samples compared to the control. Lipid-containing vacuoles are indicated with arrows. (all images x 20 magnification, representative of n=3).

HS5 cells that were in co-culture with U266B1 and not exposed to chemotherapy had a noticeable reduction in their ability to produce adipocytes (figure 4.32A). HS5 cells that were not exposed to drug had a different morphology to the independent cultures with their cell bodies appearing angular with long thin projections. A small number of these cells had an indication of lipid containing vacuoles but were severely altered compared to those cells that had been cultured alone.

Melphalan, as with the alone cultures, caused serious damaging effects to the morphology of the HS5 cells when in co-culture with U266B1, with no lipid containing vacuoles evident (figure 4.32B). Also no lipid containing vacuoles were present in HS5 cells previously exposed to thalidomide (figure 4.32C). However, small numbers of lipid containing vacuoles evidencing adipocyte formation were observed in those cultures previously exposed to lenalidomide (figure 4.32D). The altered morphology and reduction in adipocytes formation was also observed in those cultures previously exposed to carfilzomib, where only minor lipid containing vacuoles were visible (figure 4.32E). However, as with the osteogenic differentiation of HS5 cells, treatment with bortezomib in co-culture with U266B1 had severe effects on the morphology and adipogenic differentiation of these cells. These cells also failed to differentiate and had become lymphoblast-like, dissociating from the well and were thus removed from the well during the final stages of staining.

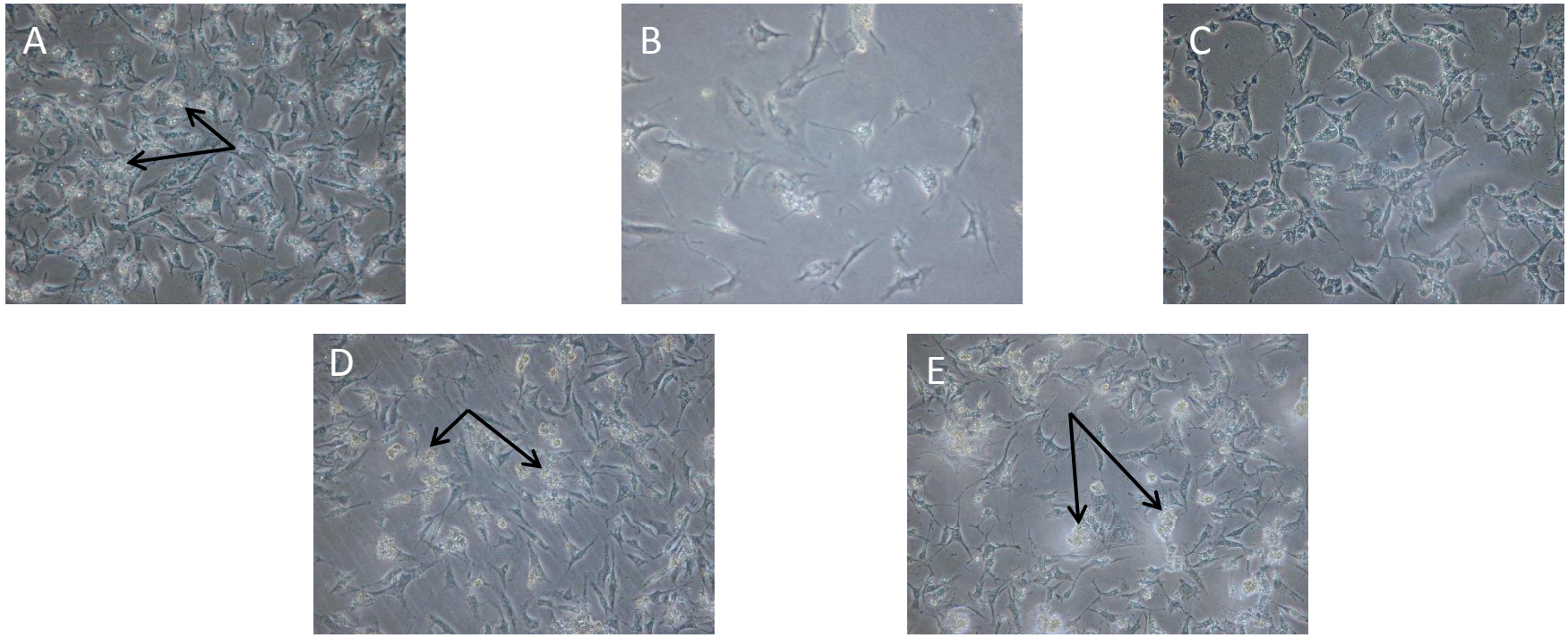


Figure 4.32 Images depicting HS5 cells co-cultured with U266B1, differentiated along adipogenic lineages following chemotherapy exposure. HS5 cells were seeded at 5 x 10^4 per well, with 5 x 10^5 U266B1 cells in an insert together in 1.5 mls adipogenic medium and were maintained by complete medium replacement every 3 or 4 days and examined at day 21 following oil red O staining. A – HS5 cells untreated, B – Melphalan (32.8 µM), C – Thalidomide (200 ng / ml), D – Lenalidomide (4 µM), E – Carfilzomib (13.8 nM). Adipogenic differentiation was significantly reduced as a result of a co-culture with U266B1 cells as evidenced by the lack of lipid-containing vacuoles and their severely altered morphology. Adipogenic differentiation appeared to be adversely affected further in all other chemotherapy treated samples. Lipid-containing vacuoles are indicated with arrows. (All images x 20 magnification, representative of n=3).

4.3.4 HS5 CD expression following chemotherapy exposure

To examine the effects of chemotherapeutic agents (melphalan, thalidomide, lenalidomide, bortezomib and carfilzomib) on CD markers 105, 73, 45, 34 and 14 on HS5 cells, flow cytometry was used. HS5 cells are known to be positive for CD105 and CD73 and negative for CD45, CD34 and CD14 (Ramos *et al*., 2016) which characterise their mesenchymal origin (Dominici *et al*., 2006). Representative histogram plots for each of these markers against an isotype control is illustrated in figure 4.33. These findings concurred with that of Ramos *et al*. (2006) with HS5 cells being positive for CD73 and CD105 and lacking the expression of CD14, CD34 and CD45.

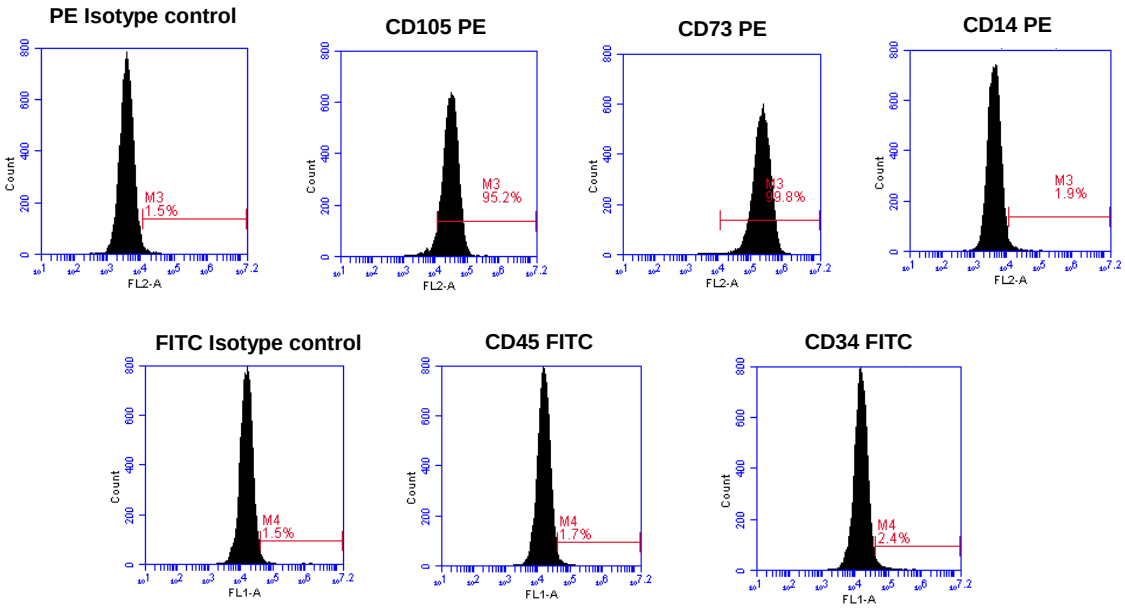


Figure 4.33 Representative histogram plots of CD markers on HS5 cells not exposed to chemotherapy. Ten thousand cells were analysed through the gate. Cell debris was not included in the collection gate and thus was not included in the analysis. The red indicator was placed so that 1.5% of cells were positive when stained for the isotype control. Any fluorescence detected above this was taken as positive for that marker. HS5 cells did not express the CD markers CD14, CD34 and CD45 and were positive for CD73, CD105.

To investigate whether expression of these CD markers was affected as a result of the exposure to chemotherapy, HS5 cells were cultured alone and treated with chemotherapeutic agents for 1 hr. Cells were sampled for flow cytometry at 72 hrs post exposure. The median fluorescence intensity (MFI) of each of the surface markers investigated is presented in figure 4.34. Isotype controls were included to show levels of nonspecific binding of Ab.

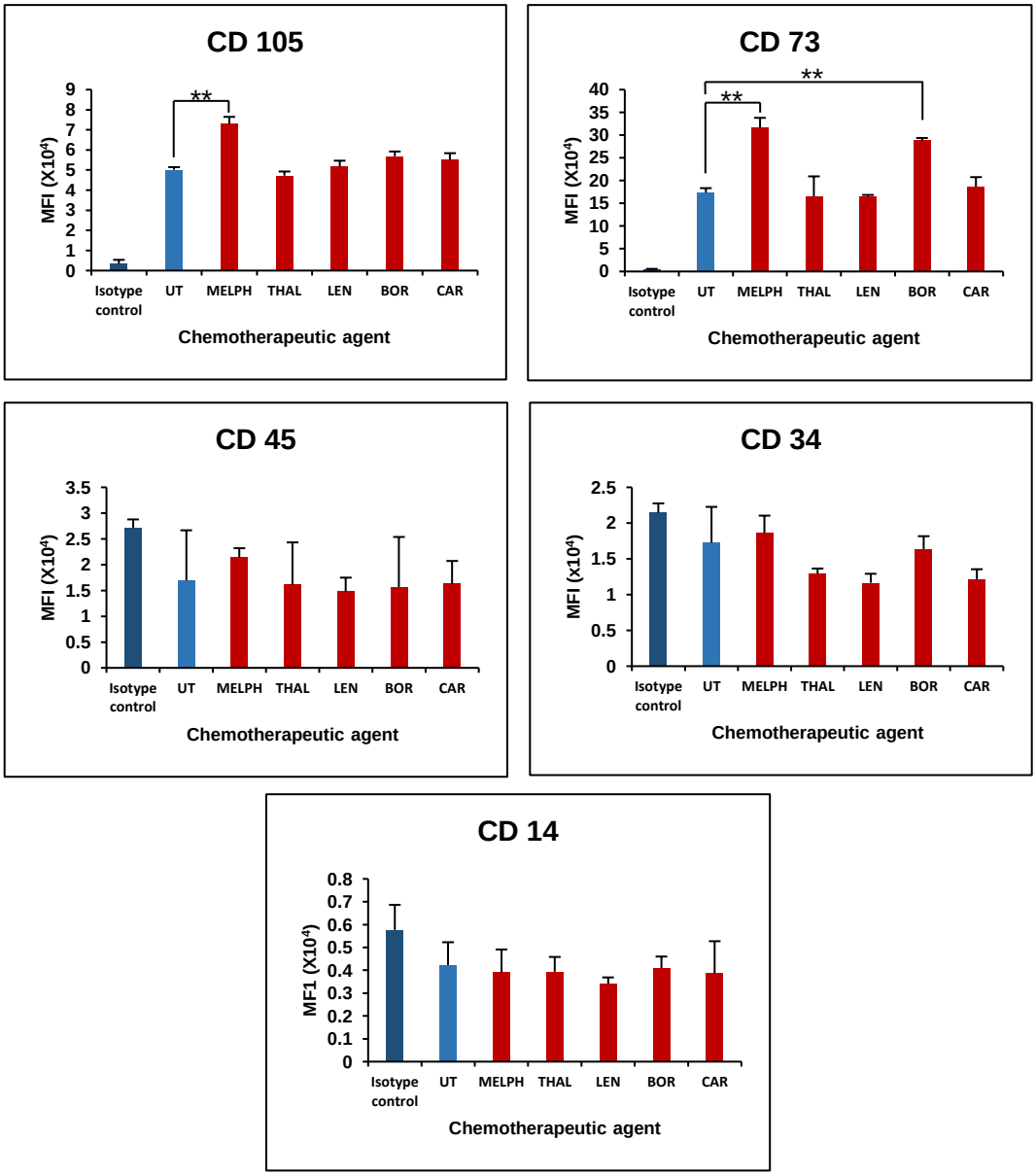


Figure 4.34 Median fluorescence intensity (MFI) of CD markers on HS5 cells following 1 hr exposure of chemotherapeutic agents. HS5 were stained with relevant CD marker antibodies at 72 hrs post exposure to chemotherapeutic agents. Cell debris was not included in the collection gate and thus was not included in the analysis. Ten thousand cells were analysed through the gate. HS5 cells were shown to have increased expression of CD 105 and CD 73 following melphalan exposure. Increased expression of CD 73 was also observed following bortezomib treatment. Results are expressed as mean ± SD (n=3) (** p<0.01).

HS5 cells were found to be positive for CD105 and CD73 and negative for CD45, CD34 and CD14 when compared to their relevant isotype control. Subsequent analysis of samples compared untreated cells against cells exposed to chemotherapy. HS5 cells that were treated with melphalan had increased levels of CD105 expression compared to the untreated control. Melphalan exposed HS5 cells had significantly higher levels of MFI compared to the untreated ($p<0.01$) 72 hrs post exposure to drug. Thalidomide and lenalidomide exposed HS5 cells expressed levels of CD105 consistent with that of the untreated control. Bortezomib and carfilzomib had slightly elevated MFI compared to the untreated but were not significant ($p>0.05$).

The levels of CD 73 expression on the surface of the HS5 membrane after treatment with melphalan, were significantly higher ($p<0.01$) than those that were untreated. This expression was also found to be significantly higher in HS5 cells exposed to bortezomib compared to the untreated cells ($p<0.01$). Each of the other chemotherapeutic agents were found to give rise to MFI levels similar to that of the untreated control.

Levels of MFI of CD45, CD34 and CD14 in untreated exposed samples were all lower than the isotype control indicating that these cells were negative for the expression of these CD markers. There was also no significant difference in samples previously exposed to chemotherapy compared to the untreated in each of these samples.

4.3.5 ELISA

4.3.5.1 Levels of IL-6 in the MM model

Following development of a sandwich ELISA for IL-6, samples from primary MSC, HS5 and U266B1 cells were investigated for levels of IL-6. Supernatants were collected from previous experiments whereby MSC/HS5 and U266B1 cells were cultured separately as well as co-cultured together. These were stored at -80°C until required. All ELISA experiments were conducted on supernatants from samples 72 hrs post exposure to chemotherapy and from samples that were untreated.

4.3.5.2 IL-6 measured in MSC following exposure to melphalan

Primary MSC were either cultured alone or co-cultured with U266B1 cells and exposed to melphalan (32.8 μM) for 1 hr. Cells were washed free of drug and at 72 hrs post exposure supernatant from both cultures was removed. MSC that were cultured independently and left untreated expressed a mean concentration of IL-6 of 793 ± 52 pg / ml (figure 4.35). MSC that were cultured independently and treated with melphalan expressed a mean concentration of IL-6 of 771 ± 120 pg / ml. There was no significant difference in the concentrations of IL-6 in the supernatant of these samples ($p>0.05$) thereby indicating that melphalan does not have an effect on IL-6 expression from primary MSC.

In contrast, concentrations of IL-6 in the supernatants of a co-culture (separated by an insert) of MSC and U266B1 cells were notably higher (figure 4.35). Co-cultures of MSC and U266B1 cells that were untreated contained a mean concentration of IL-6 of 1077 ± 118 pg / ml and were significantly higher than the concentration of IL-6 from MSC cultured alone ($p<0.05$). Melphalan treated co-cultures of MSC and U266B1 cells had reduced concentrations of IL-6 (998 ± 88 pg / ml) compared to the untreated co-culture but this was not significant ($p>0.05$).

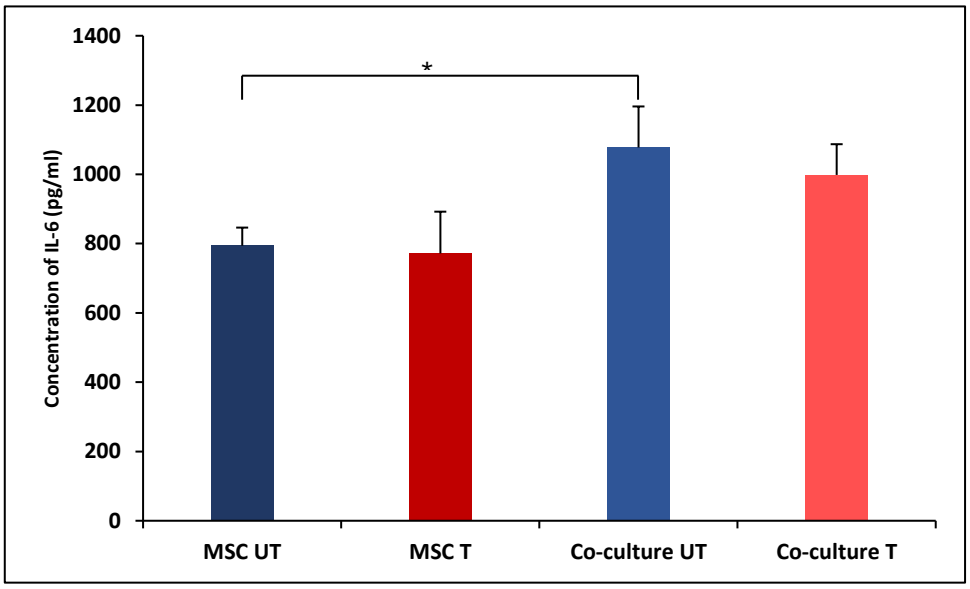


Figure 4.35 Concentration of IL-6 in primary MSC following exposure to melphalan when cultured alone or in co-culture (non-contact) with U266B1 cells. Supernatants of MSC that were cultured alone contained a significantly lower concentration of IL-6 compared to a co-culture of MSC and U266B1. Melphalan did not have a significant effect on the concentration of IL-6 in MSC alone cultures and co-cultures of MSC with U266B1 cells. Results are expressed as mean ± SD (* $p<0.05$, n=3).

4.3.5.3 Levels of IL-6 in MSC and HS5 cells when cultured alone

The concentration of IL-6 in primary MSC and the cell line HS5 when cultured alone was compared (figure 4.36). MSC and HS5 cells were cultured independently in DMEM/F12 complete medium for 72 hrs. Supernatants from primary MSC were found to contain a mean concentration of IL-6 of 872 ± 21 pg / ml which was significantly higher than the mean concentration of IL-6 in stromal cell line HS5 (414 ± 152 pg / ml) ($p<0.01$).

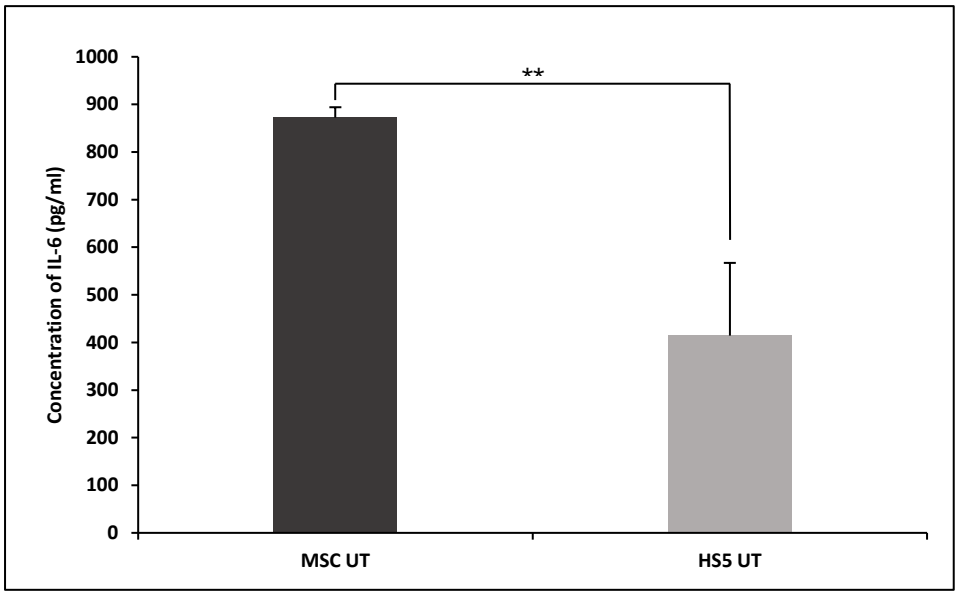


Figure 4.36 Comparison of the levels of IL-6 in primary MSC and HS5 cells when cultured independently and left untreated. Cells were cultured for 72 hrs before the supernatant was removed. Significantly higher concentrations of IL-6 were found in primary MSC compared to the stromal cell line HS5. Results are expressed as mean ± SD (**$p<0.01$, n=3).

4.3.5.4 IL-6 measured in HS5 cells following exposure to chemotherapy when cultured alone

HS5 cells were cultured alone and exposed to either melphalan (32.8 µM), thalidomide (200 ng / ml), lenalidomide (4 µM), bortezomib (500 nM) or carfilzomib (13.8 nM) for 1 hr. A control group of cells were cultured that were untreated. Cells were washed free of drug and remained in complete DMEM/F12 culture medium before the supernatant was removed 72 hrs post exposure to chemotherapy. HS5 cells that were left untreated had a mean concentration of IL-6 of 414 ± 65 pg / ml (figure 4.37). Melphalan exposed HS5 cells did not significantly alter the concentration of IL-6 ($p>0.05$) and expressed levels of IL-6 that were similar to the control (406 ± 42 pg / ml). Each of the other agents all notably reduced the concentration of IL-6 as measured in the supernatants from HS5 cells. However, lenalidomide was the only chemotherapeutic drug to significantly lower the concentration of IL-6 (254 ± 108 pg / ml) compared to the untreated control ($p<0.05$).

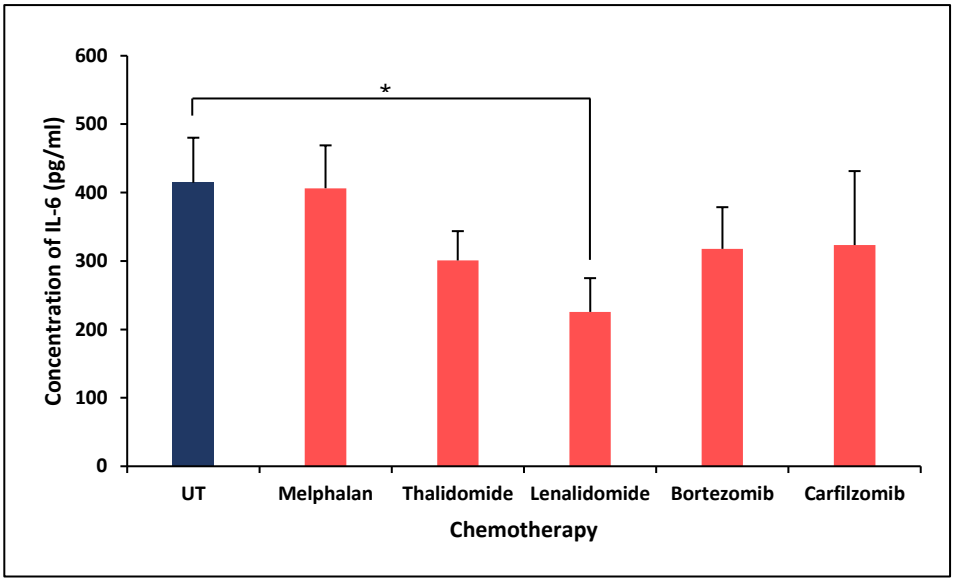


Figure 4.37 Concentration of IL-6 in the supernatant of HS5 cells when cultured alone following exposure to chemotherapy. Supernatants were recovered from cell cultures 72 hrs post exposure to chemotherapy. Concentrations of IL-6 were determined using the previously developed IL-6 ELISA. Lenalidomide was the only agent that resulted in a significant decrease in the concentration of IL-6 in HS5 cells. Results are expressed as mean ± SD (* $p<0.05$, $n=3$).

4.3.5.5 IL-6 measured in U266B1 cells following exposure to chemotherapy when cultured alone

U266B1 cells were cultured alone and exposed to either melphalan (32.8 µM), thalidomide (200 ng / ml), lenalidomide (4 µM), bortezomib (500 nM) or carfilzomib (13.8 nM) for 1 hr. A control group of cells were cultured that were not exposed to drug. The supernatant from each culture was removed 72 hrs post exposure to chemotherapy.

U266B1 cells that were not exposed to chemotherapy had a mean concentration of IL-6 of 646 ± 60 pg / ml (figure 4.38). As with primary MSC and HS5 cells, melphalan did not significantly alter the concentration of IL-6 (574 ± 33 pg / ml) in U266B1 cells compared to the control. Treatment with thalidomide reduced the concentration of IL-6 to a mean 432 ± 60 pg / ml which was significantly lower than the untreated ($p<0.05$). Supernatants from U266B1 cells exposed to lenalidomide had a mean concentration of 404 ± 138 pg / ml which was significantly lower than the untreated ($p<0.05$). Bortezomib further decreased levels of IL-6 (336 ± 173 pg / ml) in U266B1 cells which was significant compared to the control ($p<0.05$). Carfilzomib exposure also produced a significant decline in the concentration of IL-6 (341 ± 84 pg / ml) in U266B1 alone cultures compared to the untreated ($p<0.01$).

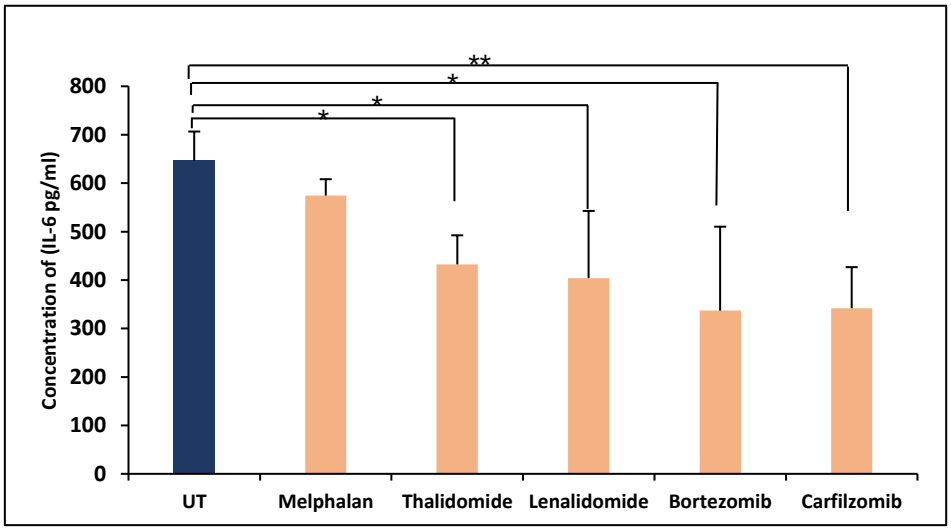


Figure 4.38 Levels of IL-6 in U266B1 cells following exposure to chemotherapy when cultured alone. Immunomodulatory agents and proteasome inhibitors significantly reduced the concentration of IL-6 in U266B1 cells when cultured alone. A sandwich ELISA was performed on supernatants recovered from U266B1 cultures 72 hrs post exposure to chemotherapy. Results are expressed as mean ± SD (* $p<0.05$, ** $p<0.01$, n=3).

4.3.5.6 IL-6 measured in a non-contact co-culture of HS5 and U266B1 cells

HS5 and U266B1 cells were co-cultured together, separated by an insert. Both cell compartments were exposed to chemotherapy (melphalan / thalidomide / lenalidomide / bortezomib / carfilzomib) for 1 hour while in co-culture or left untreated. Cells were co-cultured for 72 hrs before the supernatant was removed and stored at -80 °C, until IL-6 levels were determined using the developed IL-6 ELISA. Concentrations of IL-6 were significantly higher when HS5 and U266B1 cells were in co-culture (1027 ± 93 pg / ml) compared to HS5 (473 ± 152 pg / ml) ($p<0.01$) and U266B1 (646 ± 60 pg / ml) ($p<0.01$) cells cultured alone and left untreated (figure 4.39A).

Each of the chemotherapeutic agents caused a reduction in the concentration of IL-6 in co-cultures of HS5 and U266B1 cells compared to the untreated (figure 4.39B). However, levels of IL-6 following exposure to melphalan (908 ± 64 pg / ml) and thalidomide (803 ± 144 pg / ml) were not significantly lower than the control. Treatment of the co-culture with lenalidomide produced the most significant effect compared to the untreated ($p<0.001$), reducing the concentration of IL-6 within the co-culture to 535 ± 19 pg / ml. Bortezomib treatment of the co-culture reduced the concentration of IL-6 to a mean concentration of 609 ± 149 pg / ml, which was significantly lower than the untreated control ($p<0.05$). Furthermore, carfilzomib was also found to reduce levels of IL-6. The mean concentration of IL-6 in the co-culture of HS5 and U266B1 following exposure to carfilzomib was 566 ± 96 pg / ml, which was significantly lower than the control ($p<0.01$).

Taken together these results indicate that IL-6 secretion is increased when HS5 and U266B1 cells are in a non-contact co-culture and this significant increase may contribute to the promotion of the proliferation and survival of MM cells.

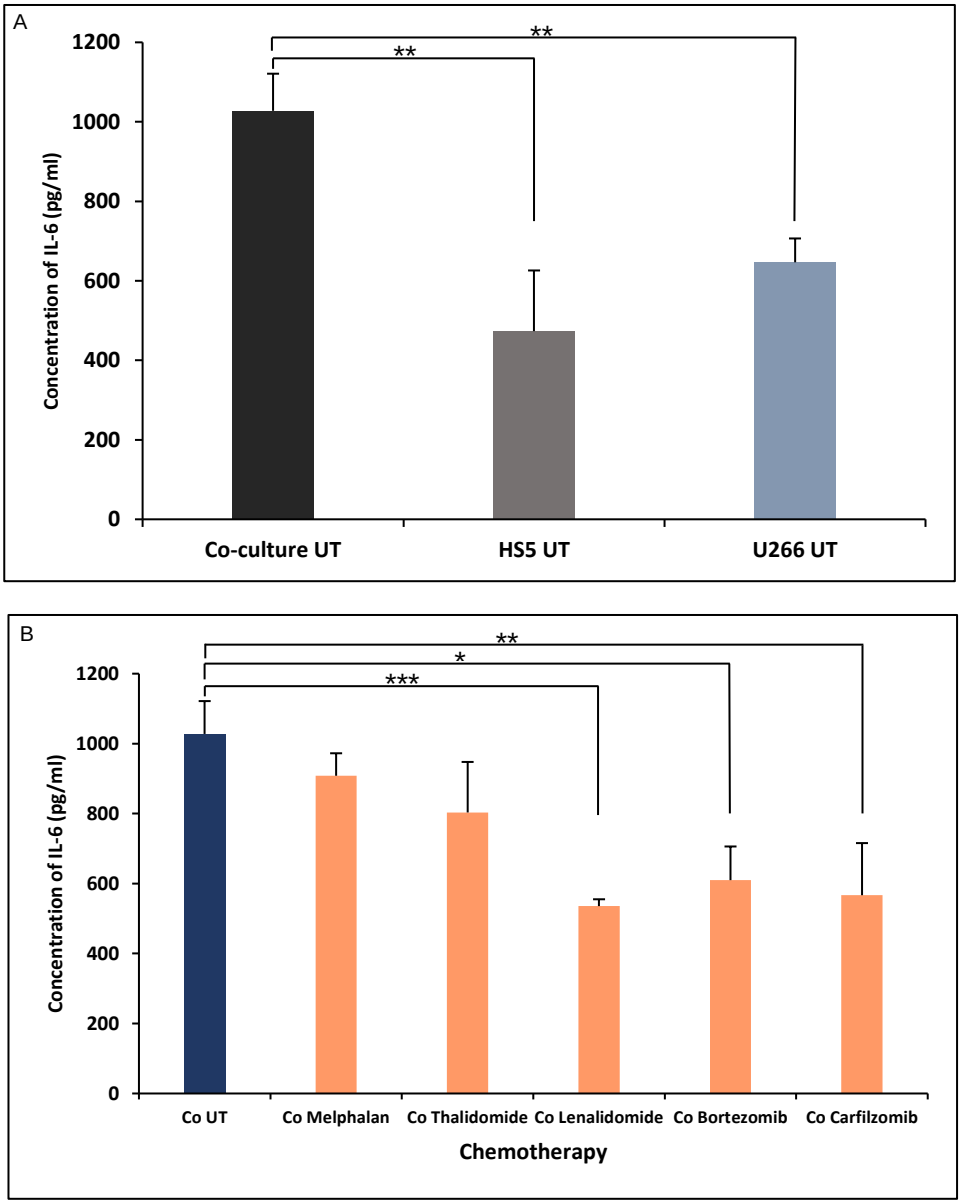


Figure 4.39 Concentration of IL-6 in a non-contact co-culture of HS5 and U266B1 cells compared to when cells are cultured alone (A) and concentration of IL-6 in a non-contact co-culture of HS5 and U266B1 cells following exposure to chemotherapy (B). The concentration of IL-6 was higher in the co-culture of HS5 and U266B1 cells compared to when cultured alone. Levels of IL-6 were lower in each of the co-cultures that were exposed to chemotherapy. Proteasome inhibitors bortezomib and carfilzomib significantly reduced the concentration of IL-6. Co-cultures that were exposed to lenalidomide had the most reduced concentrations of IL-6 compared to the control. Results are expressed as mean ± SD (*p<0.05, **p<0.01, *** p<0.001, n=3).

4.4 Discussion

Several studies have demonstrated that the BM microenvironment provides a permissive niche for tumour cell growth, with interactions between MM cells and the BM microenvironment contributing to bone destruction by deregulation of soluble factors and cell to cell cross talk (Roccaro *et al*., 2014; Gunn *et al*., 2006; Dankbar *et al*., 2000). However, the role of MSC in MM has not been fully elucidated with controversy regarding their function as tumour promoters (Zhang *et al*., 2013) or suppressors (Ho *et al*., 2013). Repeated reports of clinical studies have addressed MSC in MM, describing abnormalities of these cells in patient samples (Jurczyszyn *et al*., 2015; Andre *et al*., 2013 and Corre *et al*., 2007). With the BM offering protection to MM cells it is not known if MM cells provide protection to the BM microenvironment or whether they compromise its functionality. The results presented here assemble a scenario where MM cells profoundly affect their (adjacent) microenvironment and vice versa.

4.4.1 Cytotoxic assessment of MM model

It has long been established that cell membrane integrity is fundamental in distinguishing live and dead cells (Kroemer *et al*., 2009). A number of different methods have been developed to measure cell viability, including trypan blue, neutral red and MTT tetrazolium assay, each technique having their own benefits and disadvantages (Riss *et al*., 2015; Avelar-Freitas *et al*., 2014; Fotakis and Timbrell, 2006). For this study, trypan blue was chosen for its ease of use and speed of producing results, considering the high number of cell counts that needed to be performed. Trypan blue staining is a well-recognised measure of cell viability. Cells with an intact membrane are impermeable to the dye and are considered viable, whereas compromised cell membranes allow the dye to enter, giving the cell a distinctive blue colour when observed under light microscopy (Tran *et al*., 2011). However trypan blue is unable to indicate senescent cells or metabolically inactive cells and may overestimate cell viability (Fuller *et al*., 2004), although it is necessary to mention that in this study, trypan blue showed considerable consistency between independent experiments.

MSC and HS5 stromal cells along with U266B1 cells were compared in order to understand the changes in cell viability of the BM microenvironment during MM progression. The total cell numbers and viability of primary MSC, HS5 stromal and U266B1 cell lines following chemotherapy at each clinical dose was compared. Furthermore when studying the effects of melphalan in the *in vitro* MM model, U266B1 melphalan resistant cells were also used to study the possible effects of a resistant cell line within the model. The emergence of drug-resistance during the treatment of MM is a major complication for effective chemotherapy. Although drug resistance can be the result of several different physiological and biological processes in the body, *in vivo* research is not always a feasible approach and is thus limited with animal experimentation or where appropriate, human samples can be tested. In some research, patients' samples were used to study drug resistance in MM. However patient samples were not available for this study and so an appropriately developed drug resistant cell line was used.

For several decades, melphalan has been the gold standard to treat MM and has been shown to induce DNA damage (Dimopoulos *et al*., 2007), thus inducing a genotoxic and cytotoxic affect. This study has shown that at the clinical dose of 32.8 μM melphalan, caused a significant loss in viability of primary MSC and HS5 when cultured alone compared to the untreated cells at 72 hrs. Total cell numbers were affected, mainly at 48 hrs post exposure followed by a recovery at 72 hrs. MSC and HS5 not exposed to melphalan had increased proliferation at each time point with a consistent viability across the time course. These findings correspond with that of Kemp and colleagues (2010) which showed that melphalan disrupts the replicating ability of MSC, which corresponds with reductions in proliferative capacity of MSC harvested from patients who had received prior chemotherapy treatment. Furthermore these findings also correspond with the low numbers of BM stromal osteoprogenitors in patients after chemotherapy exposure *in vivo* (Banfi *et al*., 2007).

There was a severe drop in sensitive U266B1 cell viability at each time point along with a reduction in cell numbers. This study used a single dose of melphalan for 1 hr. However to further improve the cytotoxic effects of

melphalan, previous research has investigated the effects of melphalan on the RPMI 8226 cell line and indicated that sequential exposure to melphalan might be superior to single dose exposure (Pinguet *et al*., 1999). In contrast to the sensitive U266B1 cells, melphalan resistant U266B1 cells had improved cell numbers and viability, a finding that correlated with Salehan (2012) which reported significantly higher cell death in U266B1 melphalan sensitive cells compared to resistant cells. In order to further improve the cytotoxic effects of melphalan on MM drug combinations could be tested. A study by Chauhan *et al*. (2003), documented high intracellular concentrations of free melphalan in RPMI-8226 cells when using melphalan-flufenamide, a novel dipeptide prodrug of melphalan as compared to cells exposed directly to melphalan. This could enhance the therapeutic potential of melphalan, overcome drug-resistance, and improve MM patient outcome.

Understanding how MM affects MSC requires *in vitro* experiments and models of MSC co-cultured with MM cells. Several *in vitro* MM models have been described to study stromal cell-myeloma cell interactions, however often differing in time periods of co-culture, soluble factors or direct cell-cell contact requirements, the use of other cell types, and chemotherapy among other parameters (Reagan and Ghobrial, 2012). In this study where cells were not in direct contact, primary MSC or HS5 that were co-cultured with U266B1 and not exposed to drug, experienced a decrease in their total cell numbers over 72 hrs compared to when cultured independently. These findings reveal that disease alone disrupts the growth and viability of the BM. Damage to MSC and HS5 cells was exacerbated when in co-culture with U266B1 and exposed to melphalan, with a further reduction in numbers compared to the untreated control and had a reduction in viability at each time point noted.

In contrast the U266B1 sensitive cell compartment of the co-culture with primary MSC had an improvement in their cell numbers and viability compared to when cultured independently. This finding was also revealed when co-cultured with the stromal cell line HS5. This outcome was also noted in a recent study by Kim *et al*. (2012), which observed increased proliferation of MM cell lines and a decrease in the rate of apoptosis of MM

cells in the presence of either MSC and/or macrophages compared to a cell line-only control when in direct co-culture and unexposed to drug. Additionally this study has revealed that after exposure to melphalan there was an improved viability of sensitive and melphalan resistant cells, co-cultured with primary MSC or HS5 when compared to independent culture. Taken together, these findings reveal that U266B1 cells co-cultured with MSC/HS5 provides protection from the cytotoxic effects of melphalan. Moreover they confirm the finding that MSC protect MM cells from chemotherapy (Castells *et al*., 2012; Reagan and Ghobrial, 2012) to the detriment of the BM stroma.

Immunomodulatory agents including thalidomide and lenalidomide are widely used in the treatment of MM. Thalidomide was introduced as a therapy for patients with MM due to its anti-angiogenic properties (D'amato *et al*., 1994). In recent years, lenalidomide has become the lead immunomodulatory agent in MM therapy as a single agent overcoming the non-haematological adverse effects of thalidomide (Mazmuder and Jagannath, 2006). Like thalidomide, lenalidomide exerts pleiotropic effects, which include immunomodulatory, antiangiogenic and antineoplastic activities (Galustian and Dalgleish, 2009). However, there is a lack of a strong cytotoxic effect of these compounds on tumour cell lines or primary tumour cells, with a few exceptions as published previously (Verhelle *et al*., 2007; Mitsiades *et al*., 2002).

Thalidomide and lenalidomide caused an initial reduction in viability of HS5 when cultured alone. However, this improved over the course of 72hrs and was not significantly different to the control. Lenalidomide however did cause a reduction in the total numbers of HS5 cells. Cell numbers of U266B1 cells following exposure to either of these agents was not greatly affected and was even improved when in co-culture with HS5 cells. This paradoxically indicates that lenalidomide and thalidomide favours the growth of MM cells, in a cell line model of MM. A recent study by Bam and colleagues (2015) also reported that lenalidomide stimulated the growth of the MM cell line ARP1 when in co-culture with complete BM from healthy patients. Furthermore the expression of TNF-α was found to be increased in MM cells following lenalidomide exposure (Maiga *et al*., 2013), an observation that

raises important questions for therapeutic approaches incorporating the agent, due to its role in supporting MM cell growth (Hideshema *et al*., 2001). Additionally, when investigating these immunomodulatory agents under bystander conditions no significant changes in HS5 or U266B1 cells' viability was observed. These results, further reiterate the lack of cytotoxic effect of these agents on HS5 and U266B1 cells. However Zhu *et al*., (2008) found an increase in the cytotoxicity of lenalidomide in tumour-derived cell lines K562, Raji and PC-3 when in a co-culture with peripheral blood mononuclear cells when natural killer cells were present. It may therefore be more appropriate to introduce immune cells such as natural killer cells, into this co-culture model, to further investigate the potential cytotoxic effects of these agents in MM.

Proteasome inhibition has emerged as an important therapeutic strategy in MM. Bortezomib was the first proteasome inhibitor approved for the treatment of relapsed/refractory MM (Kane *et al*., 2003) as it was shown to directly inhibit the proliferation of MM cells and induce apoptosis (Mitsiades *et al*., 2002). Bortezomib has also been shown to inhibit the adhesion of MM cells to BM-MSC (Kim *et al*., 2015). Carfilzomib is a next-generation proteasome inhibitor that selectively and irreversibly binds to the constitutive proteasome and immunoproteasome, resulting in sustained proteasome inhibition (Khan and Stewart, 2011). It has been used to treat patients who are intolerant or resistant to both bortezomib and lenalidomide (Wang *et al*., 2014b).

Bortezomib and carfilzomib treated HS5 cells cultured alone experienced a significant fall in their total cell numbers and viability after 72 hrs post exposure, a finding supported by the results of Kim *et al*., (2015) who found that bortezomib inhibits the proliferation of BM-MSC from healthy and MM patients. At the time of writing, this is the first study demonstrating an inhibition in the proliferation on HS5 cells by carfilzomib, although activity of carfilzomib is well documented and preclinical studies have demonstrated pleiotropic cellular effects comparable to those of bortezomib (Parlati *et al*., 2009; Kuhn *et al*., 2007).

The effects of bortezomib on HS5 were further exacerbated when in co-culture with U266B1 cells with viability of HS5 cells below 30% at 72 hrs. These results suggest that bortezomib can affect HS5 cells under both normal and pathological circumstances such as an *in vitro* MM model. Interestingly however, Perez *et al*. (2010) reported no significant cytotoxicity of bortezomib when stromal cells were in direct contact with MM cells. This suggests that the BM stroma is further protected through the adhesion of these cells, whereas without direct cell-cell contact, the stroma is more susceptible to cell death by bortezomib. In contrast carfilzomib numbers and viability, although reduced, were not as low as when cultured independently. Currently no published literature has reported the cytotoxic effects of these agents on HS5 or MSC when in co-culture with MM cells. However the vast array of soluble factors released from MM cells that interact with MSC clearly contributes to the BM stroma biology chemoresistance.

U266B1 cells cultured independently and exposed to proteasome inhibitors experienced a reduction in cell numbers and viability throughout the course of the experiment. This finding was to be expected as it has previously been documented that both bortezomib and carfilzomib inhibit the growth of MM cell lines and patient-derived MM cells (Kuhn *et al*., 2007; Hideshima *et al*., 2001). U266B1 cells exposed to bortezomib in a co-culture with HS5 had reduced cell numbers and viability. However, this appeared to plateau between 48 and 72 hrs and cell viability was above 50% as opposed to those cultured alone. These findings correlated with that of a recent study by Bam *et al*. (2015) that reported similar cytotoxic effects of bortezomib when MM cells were cultured alone and in a BM co-culture model. Treatment with bortezomib resulted in growth inhibition of all OPM2 and ARP1 MM cell lines when cultured with whole BM from healthy donors (Bam *et al*., 2015). Furthermore MSC from MM patients have been demonstrated to increase NF-κB activity leading to drug resistance in MM cells via a proteinaceous secreted factor (Markovina *et al*., 2010). In contrast to bortezomib, U266B1 cells exposed to carfilzomib in a co-culture with HS5 had improved cell numbers and viability compared to when administered to U266B1 cells cultured alone. A study by Gupta and colleagues (2013) reported that

carfilzomib caused significantly more cytotoxicity in chronic lymphocytic leukemia (CLL) cells alone as compared to CLL cells in culture with HS5. This could be due to soluble factors released from HS5 such as IL-6 or IL-10 which may override the usual pro-apoptotic and anti-proliferative pathways activated as a result of carfilzomib treatment (Kuhn *et al*., 2007).

The bystander effect describes the ability of cells affected by a chemotherapeutic agent or radiation to convey manifestations of damage to other cells neither directly targeted by the agent nor necessarily susceptible to it (Savu *et al*., 2015). Primary MSC that were never exposed to melphalan and cultured with melphalan treated U266B1 indicated that damage can occur in MSC even when never exposed to drug. However as this study has also demonstrated that MSC have a reduced viability when in co-culture, it is unclear if the cytotoxic effects seen here are as a result of melphalan or due to being in culture with U266B1 cells. When assessing the effects of a possible bystander effect of melphalan on U266B1, it was interesting to note that at 72 hrs the viability of the U266B1 cells was significantly improved in those both directly and indirectly treated as opposed to the MSC. This further concurs with others that MSC supports MM growth and survival (Zahedi *et al*., 2016; Kim *et al*., 2012) as well as providing protection from melphalan treatment to its own detriment.

When investigating the bystander effect, the cytotoxic effects of bortezomib on U266B1 cells were reduced when placed in a culture with previously untreated HS5 cells, a finding that was also observed following carfilzomib treatment; thus conferring some protection from the HS5 to the U266B1 cells. This finding concurred with that of Xu and colleagues (2012) that demonstrated that MSC protect MM cells against bortezomib-induced apoptosis. However U266B1 cells that were never exposed to bortezomib had decreased proliferation and viability as a result of a culture with previously treated HS5, indicating a bystander effect between the two cell types. HS5 cells that were treated with bortezomib or carfilzomib and cultured with untreated U266B1 cells had reduced cell numbers and viability similar to when these cells were cultured alone. In contrast, no cytotoxic effects were observed in U266B1 cells that were not exposed to carfilzomib

and then cultured with previously exposed HS5 cells. Thus indicating that a bystander effect may be drug specific. These findings indicate that HS5 cells do not release factors that are detrimental to U266B1 cells following carfilzomib exposure. Taken together these findings demonstrate HS5 promote MM cell growth but also show that this is to their own detriment. Moreover MM cells 'hijack' MSC and form a major component of the tumour microenvironment regulating stem cell behaviour and gain protection from chemotherapy (Roodhart *et al*., 2011; Corre *et al*., 2007). As the majority of the literature demonstrates that adhesion of MM cells to BM cells provides protection to MM from chemotherapies (Castells *et al*., 2012; Azab *et al*., 2009; Kobune *et al*., 2007), this study has indicated that cell adhesion is not a prerequisite for chemoresistance.

These data shed light on a fundamental, but previously un-investigated role of MSC interactions with MM cells and suggests that the BM stroma may play an altruistic role in the progression of MM. This research reveals the importance of studying the effects of novel agents such as proteasome inhibitors not only on the tumour cells directly, but also within the BM microenvironment. These findings demonstrate a tropism of MSC and HS5 towards the MM cell line U266B1. Furthermore this research also reveals that cell-cell contact is not essential to provide protection of both the BM and MM cells against chemotherapy which had previously been reported (Hao *et al*., 2011; Shain and Dalton, 2001). The improved viability of U266B1 cells when in co – culture (as opposed to the monocultures) following exposure to drug indicates that U266B1 cells were considerably influenced by the interaction with HS5, most likely due to the release of several growth factors such as IL-6 by HS5 that promote MM cell growth and survival (Gado *et al*., 2000; Cheung and Ness, 2002).

4.4.2 HS5 Morphology following chemotherapy exposure

An alternative source of MSC is the human BM-derived immortalized stromal cell line HS5. HS5 cells are a homogenous adherent cell line that, under normal physiological conditions, exhibit a fibroblast-like appearance similar to that of primary MSC. As with MSC, HS5 cells have a membrane that is

crucial to the fate of the cell, being involved in a number of cellular functions including cell signalling (Delk and Farach-Carson, 2012) and cell adhesion (Buehler, 2015). Understanding the shape and structure of these cells following chemotherapy or exposure to disease is important as any changes in their structural properties could cause disruptions in the cells' normal functional dynamics (May *et al*., 2012; Kemp *et al*., 2010). It must be noted that HS5 morphology has very rarely been described in published literature and as such, the descriptions that were seen in this context serve as a benchmark for describing HS5 morphology. Therefore, where possible, comparisons with primary MSC will be made.

Cellular morphology has been used historically as an important indicator of the cell characteristics and assesses cell quality (Matsuoka, *et al*., 2013). However few studies have reported the morphology of BM-MSC and HS5 following exposure to chemotherapy. This may be due to the fact that approaches to monitor morphology have been historically qualitative and require experienced interpretation. Here HS5 cell morphology has been examined using phase contrast microscopy when cells were cultured independently or in a co-culture with U266B1 (via an insert).

HS5 cells that were cultured independently and unexposed to drug retained their expected adherent fibroblast-like morphology, of a small cell body with small projections emanating from their centre which were well organised forming a confluent layer at 48-72 hrs post seeding. This finding coincides with others that have shown related morphological structures were observed in research conducted in primary MSC (Munoz *et al*., 2012). Morphological changes to HS5 that were evident after exposure to melphalan were similar to those previously reported by Kemp *et al*., (2011) which described primary MSC as having only a slightly abnormal appearance. In reviewing the literature for this thesis no published research demonstrated the effects of immunomodulatory agents or proteasome inhibitors on primary MSC or HS5. In this present study, HS5 morphology was not significantly altered following exposure to thalidomide. However lenalidomide demonstrated disorganised clusters with stellate structures. Furthermore, the size and the cell population of HS5 exposed to proteasome inhibitors exhibited a significantly

altered morphology compared to that of the untreated, with an increase in stellate processes and a failure of cells to adhere to the plate. The failure of cells to adhere correlates with a finding by Cao and colleagues (2008) who reported a significant reduction in the adhesive rate of MSC post-adjuvant chemotherapy.

In the co-culture setting with U266B1 cells, a lack of the normal morphology of HS5 was evident. It was clear that HS5 in co-culture and not exposed to drug had failed to reach confluence after 72 hrs, which was not previously seen when cultured alone. This finding further suggests that the cross-talk via soluble factors between these two cell types even without chemotherapy is damaging to the HS5. The HS5 cells had lost their ordered cellular phenotype that was seen in the monocultures and had become loosely aggregated; a finding that concurred with Windus *et al* (2013), who looked at the effects of a prostate cancer cell line (PC3) when co-cultured with HS5. Through soluble and contact mediated mechanisms, PC3 and HS5 cells reciprocally interact, leading to a highly disorganised arrangement of cells characterised by long chains of stellate processes, consistent with a highly invasive phenotype (Windus *et al*., 2013). Additionally, primary MSC have been shown to demonstrate an altered morphology when in co-culture with fibro-chondrocytes, with cells initially having elongated morphology that transitioned to a circular morphology over time (McCorry *et al*., 2016). This further demonstrates that MSC phenotype can be influenced by co-culture. When chemotherapy was administered into the co-culture, the effects were further exacerbated. Each of the chemotherapeutic agents investigated here caused a range of effects including increased aggregation of cells, cells with stellate processes and reduction in cell adherence with an increase in lymphoblast like cells. The findings here, combined with the increased proliferation and viability of MM cells, suggest that HS5 cells provide positive factors that support MM cell growth at the detriment to their own morphology.

This altered morphology along with the changes in proliferative rates and viability, were likely as a result of interactions between the cells via growth factors such as IL-6 released within the co-culture structure (Bam *et al*., 2015). These findings may have important clinical implications, for the role of

MSC in secondary malignancy within the BM, as well as failure of ASCT. This study has shown that the crosstalk operating between these cells affects both HS5 and U266B1 and therefore differentiation of HS5 cells was assessed within the model.

4.4.3 HS5 Cell differentiation

A unique characteristic of MSC is the ability for them to differentiate down a number of lineages, including osteogenic and adipogenic lineages (Chamberlain *et al*., 2007). During this complex process MSC undergo extensive modifications to their morphology and cytoskeleton (Yourek *et al*., 2007 and Rodriguez *et al*., 2004). The commitment and differentiation of MSC towards an adipogenic or osteogenic lineage *in vitro* can be achieved by incubation in a medium containing several types of soluble factors (Pittenger *et al*., 1999). Here the effects of chemotherapeutic agents as well as the MM cell line U266B1 on producing these mesenchymal lineages from HS5 cells were examined.

It is known that osteolytic bone disease resulting in bone pain and pathological fractures is the most common symptom in MM (Fowler *et al*., 2011). These lytic lesions are caused by the increased activity of osteoclasts, further exacerbated by the suppression of osteoblast differentiation and function (Tian *et al*., 2003). Here the osteogenic differentiation was confirmed by staining for the bone marker ALP. Previous studies of primary MSC cultured in osteogenic conditions exhibited a change in cell shape from their usual fibroblast-like phenotype cells becoming cuboidal in shape (Rodriguez *et al*., 2004). In this investigation HS5 stromal cells were also able to differentiate with a change their normal morphology giving rise to enlarged cuboidal cell bodies that stained blue for ALP.

It is widely appreciated that MM cells insidiously overtake normal bone homeostasis to decrease osteoblastic activity and increase osteoclastic activity by altering local microenvironment cells (Habibi *et al*., 2013; Papadopoulou *et al*., 2010; Giuliani *et al*., 2009). This research identified a clear reduction in osteoblast differentiation of HS5 when in co-culture with

U266B1. Furthermore this study was carried out using a non-contact model and so evidences that the reduction in osteoblast differentiation is possible through cross talk via soluble factors between MM cells and HS5.

The effects of melphalan on MSC differentiation have not previously been reported. These results indicate for the first time that HS5 cells that were induced down an osteogenic lineage following exposure to melphalan became severely affected. HS5 cells cultured alone had an abnormal morphology with the bodies of these cells becoming enlarged with reduced ALP activity. A similar finding was also observed in HS5 cells that were co-cultured with U266B1 and exposed to melphalan.

HS5 cell differentiation appeared unaffected following exposure to immunomodulatory agents. There was a slight reduction in ALP activity as measured by the blue staining of HS5 cells cultured alone. When in co-culture with U266B1, and exposed to immunomodulatory agents there was a visible reduction in cell numbers of HS5 comparable to the untreated. Morphology of the HS5 was also similar to that of the untreated; with ALP activity evidenced by the blue staining of cells, with no apparent effect of either thalidomide or lenalidomide on osteogenic differentiation of HS5. The literature reports are conflicting as to the effect of these drugs on the differentiation of MSC. Wobus *et al*. (2012) reported no effect of lenalidomide on osteogenic differentiation of MSC, which agreed with the results of this study. However Bolomsky *et al*. (2014) reported that thalidomide and lenalidomide significantly inhibited osteoblast development in MSC *in vitro*.

The UPS pathway is one of the major pathways for protein degradation within cells and is involved in cell proliferation and survival of MM cells (Cao and Mao, 2011). A study by Garret and colleagues (2003), demonstrated that this pathway may regulate osteoblast differentiation and bone formation *in vitro* and *in vivo* in mice. Low doses of proteasome inhibitors were shown to induce osteoblast differentiation *in vitro* as well as enhance bone formation *in vitro* and *in vivo* evidenced by an increase in bone morphogenetic protein-2 (BMP-2) expression in osteoblasts (Garret *et al*., 2003). Investigating the effect of bortezomib in this study identified ALP activity in bortezomib

exposed HS5 cells cultured alone. This finding coincides with some reports that have shown an increase of ALP activity *in vivo* after bortezomib administration (Zangari *et al*., 2005; Shimazaki *et al*., 2005). Further to this, low doses of bortezomib (1.0 – 5.0 nM) have been shown to increase ALP activity *in vitro*. However the cytotoxic effects of bortezomib on both MM cell lines and patient myeloma cells were not evident at these doses (Munemasa *et al*., 2008). The results presented here suggest a scenario where ALP activity is evident in HS5 cells following bortezomib treatment at the relevant clinical dose. This correlates with studies *in vitro* and *in vivo* showing that bortezomib is associated with inducing MSC to differentiate into osteoblasts (Qiang *et al*., 2009; Giuliani *et al*., 2009). However, the morphology of HS5 cells was severely affected with an increase in lymphoblast-like cells and does not represent the typical morphology of large cuboidal shaped cells. Furthermore, when HS5 cells were directly exposed to bortezomib in a co-culture with U266B1, all cells had become lymphoblast-like, were detached from the plate and were subsequently removed during the final stages of staining. This finding has not been reported elsewhere and as the cells were not in contact, this further implies that soluble factors released from the MM cells are detrimental to the HS5. Moreover contact between MM cells and MSC may be required to promote differentiation.

Carfilzomib, like bortezomib, has exhibited potent anti-myeloma efficacy (Kuhn *et al*., 2007). The results presented here show that carfilzomib treated HS5 retain the ability to differentiate into osteoblasts in both independent cultures and in co-culture with U266B1 cells. Recently, *in vitro* studies have revealed that carfilzomib stimulates MSCs to differentiate into osteoblasts by increasing the matrix mineralization and calcium deposition (Hu *et al*., 2013). Furthermore, Li *et al*. (2014) showed that carfilzomib promoted osteogenic differentiation potential of MSC derived from MM patients.

Within the BM, the differentiation of MSCs into adipocytes or osteoblasts is competitively balanced; mechanisms that promote one cell fate actively suppress mechanisms that induce the alternative lineage (Muruganandan *et al*., 2009). The formation of adipose tissue, is crucial to the body's metabolism where adipocytes control energy balance by storing

triacylglycerol in periods of energy excess and mobilizing it during energy deprivation (Ali *et al*., 2013). Moreover adipocytes' also serve as endocrine cells secreting various cytokines called adipokines (Lau *et al*., 2005). An imbalance of adipogenesis has been associated with pathophysiological conditions, including MM (Liu *et al*., 2015). Others have reported that adipocytes directly attract MM cells by secreting chemoattractants such as MCP-1 and SDF-1α (Trotter *et al*., 2015). As such, the adipogenic differentiation potential of HS5 cells was studied in independent culture and in a co-culture with U266B1. To confirm adipogenic potential of the HS5 cells, oil red O was used to stain lipid containing vacuoles. HS5 cells that were cultured independently and unexposed to drug were able to give rise to adipocyte formation evidenced by lipid containing vacuoles. However these vacuoles were not as clearly identifiable as those seen in primary MSC in published literature whereby lipid vacuoles are stained a deep red with oil red O (Boomsma *et al*., 2007; Rivera *et al*., 2006). Additionally in the presence of U266B1 cells adipocyte formation was visible although markedly reduced with fewer lipid vacuoles visible.

As with the osteogenic cultures, melphalan caused significant effects on the ability of HS5 to differentiate. Melphalan exposed HS5 independent cultures not only presented an absence of adipogenesis but also significant structural abnormalities which were evident when exposed to melphalan in a co-culture with U266B1. This finding contrasted with that of Kemp and colleagues (2011) who reported no significant change in primary MSC adipogenesis following melphalan exposure. Adipogenic formation of HS5 in a co-culture with MM cells and melphalan has not previously been documented, although the findings presented here demonstrate significant lack of adipogenic differentiation with no lipid vacuoles present.

Thalidomide inhibited the formation of lipid containing vacuoles. A finding that correlated with Zhang *et al*. (2012) who demonstrated thalidomide inhibited adipogenesis in orbital fibroblasts from Graves' ophthalmopathy patients. Lenalidomide effects caused a reduction in the formation of adipocytes in independent cultures compared to the untreated. These findings concur with the recent research of lenalidomides' impact on

adipogenic potential of MSC. Wobus and colleagues (2012) reported no significant differences in 60% of primary MSC ability to differentiate into an adipogenic lineage following exposure to lenalidomide with 40% of samples giving rise to enhanced adipogenic differentiation. However in in this investigation the effects of U266B1 cells again further exacerbated the findings that were seen in independent cultures from both immunomodulatory agents.

The UPS has been shown to influence the formation of adipocytes, being highly active during the early stages of differentiation and decreasing in activity as the stem cells become differentiated (Sakamoto *et al*., 2010). The proteasome inhibitors, bortezomib and carfilzomib caused a reduction in adipocyte formation in HS5 independent cultures compared to the untreated cells. These findings correlated with that of Dasuri and colleagues (2011) who reported decreased lipid content following exposure to the proteasome inhibitor MG132. When exposed to bortezomib in a co-culture, as with the osteoblast differentiation, the results were further impaired and the cells had become lymphoblast-like and were lost during the final stages of staining. There were, however, a small number of adipocytes visible in the co-cultures exposed to carfilzomib.

These findings confirm that osteogenesis and adipogenesis can be induced in HS5 cells that have received chemotherapy treatment, albeit reduced compared to untreated. Additionally, co-culture with U266B1 cells provides an inhibitory effect on HS5 osteogenic and adipogenic differentiation. Importantly this study has used transwell inserts to separate the cells from direct contact. It is therefore soluble factors that have led to the significant changes in differentiation that were seen. Possible factors include IL-6 which has previously been reported to inhibit adipogenic differentiation of MSC (Pricola *et al*., 2009). Changes in HS5 differentiation as a result of this co-culture model and chemotherapy provide insights into the role of MSC in MM and their inability to promote growth of new bone or tissue.

4.4.4 HS5 CD expression after chemotherapy treatment

HS-5 stromal cells are known to express surface markers similar to that of normal primary BM-MSC. In this regard, antibodies were selected against recognized markers expressed by MSC in their membrane (CD105 and CD73) and those that are consistently negative in MSC (CD45, CD34 and CD14) (Dominici *et al*., 2006). Firstly, expression (or lack of) of these CD markers was identified in HS5 cells cultured alone which agreed with a recent publication by Ramos *et al*., (2016). HS5 cells were then cultured independently and exposed to chemotherapy for 1 hr and cells were sampled 72 hrs later.

CD105 is a membrane glycoprotein and part of the transforming growth factor - receptor complex that has been well documented to be expressed by MSC (Maleki *et al*., 2014; Mark *et al*., 2013). Its role in the prognosis, diagnosis, and treatment of MM has recently been discussed (Pappa *et al*., 2013). CD105 was found to be highly expressed in HS5 cells in this study which correlated with Harris and colleagues (2011), which also confirmed the expression of CD105 on HS5 cells. Moreover this report has demonstrated that its expression increased following exposure to melphalan whilst all other agents gave rise to MFI levels consistent with the untreated control. Braun *et al*. (2012) proposed that upregulation of CD105 on MSC was required for improved motility and attachment to the plastic. This finding would concur with the morphology of HS5 when exposed to melphalan alone where cells remained attached to the surface of the culture plate. Furthermore, CD105 has been documented to function as a co-receptor for transforming growth factor-beta (TGF-β) (Pérez-Gómez *et al*., 2010). This cytokine is known to inhibit osteogenic differentiation of MSC (Alliston *et al*., 2001), thus cells with lower CD105 expression and therefore diminished TGF-β signaling would be expected to have improved osteoblast differentiation (Lian *et al*., 2012; Maeda *et al*., 2004). Moreover a recent study by Van-Zoelen *et al*. (2016) found that TGF-β inhibited adipogenic differentiation of MSC. Therefore the high expression of CD105 following melphalan exposure may have a role in the reduced osteogenic and adipogenic capacity of these cells that was

observed in both independent and co-cultures within this study when treated with melphalan.

In this study melphalan and bortezomib treatment was found to increase the expression of CD73 on HS5 cells. CD73 is a major surface marker expressed by MSC. CD73 has been shown to be a key regulator in cancer development and drug resistance (Gao *et al*., 2014; Ujhazy *et al*., 1996). Studies have shown that CD73 expressed on stromal cells contributes substantially to tumour-induced immune suppression. Adenosine generated by CD73 expressed on tumour cells decreases the function of antitumour T cells and promotes T cell apoptosis, thereby contributing to tumour immune evasion (Wang *et al*., 2011; Jin *et al*., 2010). Therefore increased CD73 expression following melphalan and bortezomib exposure observed in this study may indicate a possible association with resistance to these agents.

CD45 (lymphocyte common antigen) is a receptor-linked protein tyrosine phosphatase that is expressed on the surface of nucleated haematopoietic cells (Altin and Sloan, 1997). It is one of the most abundant leukocyte cell surface glycoproteins and is considered to be expressed exclusively upon cells of the haematopoietic system (Yeh *et al*., 2006). In this study there was no statistical difference between samples and all had reduced levels of MFI compared to the isotype control in HS5 cells. These findings agree with that of Dominici *et al*. (2006) who identified that MSC lack the expression of CD45. Recently Ramos *et al*. (2016) also reported that HS5 cells failed to express this surface marker.

CD34 is a transmembrane sialomucin whose function remains largely unknown but has been suggested to be adhesive and/or anti-adhesive, depending on the cellular environment (Nielsen and McNagny, 2009). It was recommended as a negative marker for MSC (Dominici *et al*., 2006), and is the most commonly used marker for haematopoietic stem/ progenitor cells in clinical haematology. Additionally CD14 is a GPI linked receptor for endotoxin and its expression has been observed on human adipose tissue-derived MSC ex *vivo* (Mitchell *et al*., 2006). It has been reported to be negatively expressed on HS-5 by Roecklein and Torok-Storb (1995). Each of these

markers were found to be negative in HS5 cells in this study, with chemotherapy treatment not significantly altering the expression of these CD markers.

The present study aimed to compare the expression of MSC specific cell surface markers in HS5 cells following chemotherapy treatment *in vitro*. As described above, high expression of CD105 and CD73 may help explain the functionality of these cells during chemotherapy. Thus, the behavior of these CD markers following chemotherapy may provide useful insights into the clinical applications of these drugs in MM.

4.4.6 Expression of IL-6 in the MM model

Following development of the ELISA, supernatant samples from MSC, HS5 and U266B1 independent and co-cultures were investigated for levels of IL-6. The cytokine IL-6 has long been highlighted as a major contributor to the pathophysiology of MM (Matthes *et al*., 2016; Gerlo *et al*., 2008; Gado *et al*., 2000). One of the key roles of IL-6 in MM is to trigger the proliferation of MM cells as well as osteoclasts, promoting their survival and preventing their apoptosis (Papadopoulou *et al*., 2010; Leu *et al*., 2003; Karadag *et al*., 2000). IL-6 achieves this by inducing the activation of both the Ras/MAPK pathway, as well as the JAK/STAT pathway, which promote MM cell survival (Heinrich *et al*., 2003). Importantly, these signalling cascades triggered by IL-6 may reduce the effectiveness of conventional chemotherapeutic agents against MM cells in the BM milieu. Moreover many drug screens are performed in the absence of stromal cells in *in vitro* co-cultures and therefore may produce deceiving results (Reagan and Ghobrial, 2012).

The process of freeze-thawing a sample is believed to affect the stability of cytokines. A study by Jager *et al*. (2009) reported that a number of cytokines including IL-6 were particularly affected by multiple freeze-thawing cycles. Therefore each ELISA test was performed using a fresh aliquot of recombinant IL-6. In agreement with previous studies (Vincent and Mechti, 2004; Rougier *et al*., 1998; Roecklein and Storb, 1995) IL-6 concentrations were detected in primary MSC as well as the cell lines HS5 and U266B1

cells. However this study revealed for the first time that primary MSC secrete significantly higher amounts of IL-6 compared to the stromal cell line HS5.

Co-culture of MSC and U266B1 as well as HS5 and U266B1 had significantly higher concentrations of IL-6 in the supernatants of these samples compared to when these cells were cultured alone. This finding was in line with other studies (Xu *et al*., 2012; Zdzisińska *et al*., 2008) whereby MM cells were found to upregulate the expression of IL-6 in MSC. Lower concentrations of IL-6 were found in cultures exposed to immunomodulatory agents and proteasome inhibitors.

Melphalan, did not significantly alter the concentration of IL-6 in the supernatant of any of the cells used in this study. However this finding was to be expected as melphalan used alone has not been previously documented to inhibit IL-6. The overall mechanism of thalidomide is not completely understood with limited reports on thalidomide's ability to inhibit IL-6 secretion. One study by Li and colleagues (2002) reported that thalidomide (400 mg / ml) reduced the expression of IL-6 in the serum of MM patients *in vivo*. In this study thalidomide only significantly reduced the concentration of IL-6 in U266B1 cells that were cultured alone. In contrast, lenalidomide treatment was found to significantly lower the expression of IL-6 in HS5 cells and U266B1 cells that were cultured alone and when these cells were co-cultured together. Lenalidomide has previously been reported to inhibit IL-6 and also inhibit phosphorylation of STAT3 in a MM cell line (Spek *et al*.,2009).

MM cells have increased IκB phosphorylation and increased NF-κB activity compared with normal haematopoietic cells which is associated with increased cell survival and IL-6 secretion (Rego *et al*., 2011; Ni *et al*., 2001). As both bortezomib and carfilzomib are known to inhibit NF-κB (Mannava *et al*., 2012), it is likely that this inhibition contributes largely to the suppression of IL-6 seen in this study. Furthermore, previous reports have highlighted activation of NF-κB in BM-MSC when MM cells were in direct contact (Chauhan *et al*., 1996) whereas in this report no contact between the cells occurred. Further studies would be required to confirm the deactivation of

NF-κB in these cells although this may explain the decreased concentrations of IL-6 seen here.

Overall the increased concentrations of IL-6 within the co-culture model further highlight the importance of this cytokine in MM. Increased levels of IL-6 in co-culture combined with the increase in U266B1 cell numbers seen here reiterate its impact on MM cell growth. Furthermore these increased levels of IL-6 may also help to explain the altered phenotype and functionality of MSC and HS5 cells that was seen in this investigation. However further examination of other cytokines and soluble factors are needed to confirm their role in MSC functionality in patients with MM.

4.5 Conclusion

In the last few years the BM microenvironment has been demonstrated to play important roles in tumour pathogenesis. The data presented here illustrates damage to HS5 from clinical doses of *in vitro* chemotherapeutic treatment and concurs with other previous *in vitro* and *in vivo* studies of BM-MSC (Kemp *et al*., 2010; Mueller *et al*., 2006). MM cell line co-culture (non-contact) with HS5 protected U266B1 cells from drug-induced cell death, suggesting that interactions with the normal BM microenvironment might contribute to MM cell survival after chemotherapy exposure. However, the promoting effects of the BM stroma appear to be at detriment to their own survival. HS5 cells were found to have lower viability, altered morphology, disrupted differentiation and an increased expression of IL-6 when in co-culture with U266B1 cells. Overall, these observations assemble a scenario where U266B1 cells profoundly and mutually affect the adjacent HS5 cell phenotype and ultimately its fate *in vitro*.