Chapter 5

Genotoxic assessment of an *in vitro* model of MM

5.1 Introduction

The treatment of cancer is still largely based on the use of chemotherapeutic agents that are designed to kill or reduce proliferating cells. Through various mechanisms, these drugs interfere directly or indirectly with the cell's DNA resulting in DNA damage (Woods and Turchi, 2013). The vast majority of all genotoxic damage is subject to efficient repair by a sequence of cellular enzymes (Houtgraaf *et al*., 2006). However these repair mechanisms vary in efficiency and differ considerably between individuals (Collins *et al*., 2001). Failure to repair DNA damage effectively may perpetuate mutations and increase the future risk of malignancies (Torgovnick and Schumacher, 2015). Genetic toxicity testing is routinely performed by the pharmaceutical industry to identify potential genotoxic carcinogens using a battery of *in vitro* and *in vivo* tests recommended by regulatory agencies (Kirkland *et al*., 2011; Jena *et al*., 2002).

The BM microenvironment and its interactions with MM cells has been well documented to provide support for their growth, survival and resistance to therapeutic agents (Kawano *et al*., 2015; Abdi *et al*., 2013). Some targeted drugs such as thalidomide and bortezomib have been shown to be effective in MM. However the cancer remains incurable, with most patients eventually developing advanced, relapsing disease that is resistant to the drug(s) to which they had prolonged exposure (Abdi *et al*., 2013). This may result from protection to *in vivo* exposure by interaction of BM stromal/mesenchymal cells (BM—MSC) with MM cells. Patients with a haematological malignancy also sustain "damage" to their BM from chemotherapy exposure. However it is currently unknown if this "damage" is related to altruistic protection of MM by BM-MSC. Furthermore, whilst "damage" has been demonstrated as

compromised functionality (chapter 4), little is known of the genotoxicity to both MM and BM-MSC post-exposure to treatment.

Work in previous chapters has illustrated the cytotoxic effects of chemotherapeutic agents used in MM when administered within the developed co-culture model (chapter 4); however, many chemotherapeutic agents also exert genotoxic effects. The aim of this chapter was to evaluate the possible genotoxic effects on BM-MSC, HS5 cells and U266B1 cells, using the *in vitro* co-culture model (chapter 3) following exposure to clinical doses of chemotherapy commonly used in MM treatment. 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; (2) co-culturing HS5 and the MM cell line, U266B1 without direct physical contact; (3) co-culturing the cells following exposure to chemotherapeutic agent to one cell compartment.

5.2 Methods

5.2.1 Alkaline Comet Assay

The comet assay was used to measure DNA damage following exposure to chemotherapeutic agents (melphalan, thalidomide, lenalidomide, bortezomib, carfilzomib) at 1, 16 (for melphalan), 24 (for every other drug except melphalan), 48 and 72 hrs post exposure. All comet assays were performed as previously described in section 2.5.2.

5.2.2 Micronucleus Assay

The micronucleus assay was used following exposure to chemotherapeutic agents (melphalan, thalidomide, lenalidomide, bortezomib, carfilzomib) at 1, 16 (for melphalan), 24 (for every other drug except melphalan), 48 and 72 hrs post exposure. All micronucleus assays were performed as previously described in section 2.5.1.

5.2.2.1 Cytochalasin-B protocol

This cytokinesis-block micronucleus technique was attempted with cell cultures prepared as mentioned previously in section 2.3.1. After exposure to chemotherapeutic agents, cells were treated with 4 µg / ml cytochalasin-B at 37 ºC for 24 hr. Slides were prepared and analysed as mentioned previously in sections 2.5.1.1 and 2.5.1.2. However this method was not used further due to overt toxicity.

5.2.3 Cell cycle analysis

In order to further study the effects of the immunomodulatory agents thalidomide and lenalidomide, TK6 cells were synchronised using a thymidine double block protocol. Cells were stained with propidium iodide and analysed by flow cytometry (section 2.8.1 and 2.8.2). Following synchronisation cells were analysed for multinucleation (section 2.5.1) following exposure to thalidomide and lenalidomide.

5.2.4 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 using Microsoft Excel. An unpaired Student's t-test was used for samples compared at each time point. When comparing samples between time points a two way ANOVA was used. Differences between means were considered statistically significant if the p value was less than 0.05.

5.3 Results

5.3.1 Alkaline Comet Assay Results

The comet assay allows the quantitative analysis of DNA strand breaks, double strand breaks, DNA base damage, DNA crosslinking and DNA repair in eukaryotic cells (Collins, 2004). The comet assay was used to assess levels of DNA damage following exposure to melphalan, thalidomide, lenalidomide, bortezomib and carfilzomib for 1 hr at their clinical doses. At several time points after drug exposure, cells were harvested and analysed with the comet assay. At least 50 cells were analysed for each U266B1 and MSC experiment following melphalan exposure. All subsequent comet assays were performed with the analysis of 150 comets following updated OECD guideline recommendations (OECD guideline for the testing of chemicals, 2014). When analysing data generated using the alkaline comet assay, a number of measurements have commonly been used, including tail length, ratio of tail length to width, percentage of DNA migrated and tail moment (tail length x measure of DNA in the tail) (Tice *et al*., 2000). Percentage DNA in the comet tail (tail intensity) is the most commonly used parameter for comet analysis among laboratories and was thus recorded and used as an indicator of DNA damage in this study (Sunjog *et al*., 2013; Kumaravel and Jha, 2006). Hydrogen peroxide (H_2O_2; 50 µM for 5 mins on ice before immersing in lysis solution) was used to create a fixed amount of DNA damage which would serve as a positive control on all samples. Moreover DNA crosslinks are the critical DNA damaging effects of melphalan (Dronkerta and Kanaar, 2001) and are expressed as smaller comet tails as a result of holding the DNA together (Spanswick *et al*., 2002). When studding the effects of melphalan here H_2O_2 was used to detect the presence of DNA crosslinks. Data was analysed using excel software with the mean of three independent experiments used to create the graphs.

5.3.1.1 Melphalan

The standard first line therapy for MM is treatment with alkylating agents such as melphalan (Rodriguez *et al*., 2016). Melphalan as previously mentioned

(chapter 1) is a DNA crosslinking agent. The efficacy of melphalan to form DNA crosslinks in both MSC and U266B1 cells when in co-culture has not previously been elucidated. The levels of DNA damage induced in both sensitive and resistant U266B1 MM cell lines, BM-MSC and the stromal cell line HS5 following exposure to melphalan (32.8 μM) for 1 hr were investigated and compared. Cells were collected immediately (1 hr) as well as at 16, 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.

No significant DNA damage was detected by comet assay in MSC independent cultures and co-cultures with U266B1, following melphalan exposure at any time point (figure 5.1). DNA damage was higher at each time point following melphalan exposure in MSC independent cultures compared to untreated controls. However this was not significant, with DNA damage below 10% in all treated and untreated MSC.

U266B1 cells immediately after melphalan exposure had significantly increased DNA damage in independent cultures compared to untreated ($p<0.01$) (figure 5.1). U266B1 cells also had significantly higher DNA damage in those co-cultured with MSC ($p<0.05$). DNA damage in independent cultures was between 20 – 25% compared to between 15 – 20% in the co-culture with MSC. At 16 hrs post melphalan exposure, U266B1 cells in both independent and co-cultures showed tail retardation, evidenced by a significant reduction in DNA damage compared to H_2O_2 controls ($p<0.001$), suggesting DNA crosslink formation. DNA damage was between 4 – 6% in both independent cultures and co-cultures. At 48 hrs post melphalan exposure, U266B1 comet tail intensities increased to between 15 – 20% in both independent cultures and co-cultures, and was significantly higher compared to the untreated (independent, $p<0.01$, co-cultures $p<0.05$). At 72 hrs post melphalan exposure, comet tail intensities of the U266B1 independent cultures increased further to between 20 - 25% compared to 48 hrs and were significantly higher than the untreated cells ($p<0.001$). However in the U266B1 cells that were co-cultured with MSC there was a slight reduction in DNA damage compared to

48 hrs but this was not considered significant. DNA damage was in the region of 20 – 25% and was significantly higher compared to the untreated at 72 hrs ($p<0.05$). Representative images of MSC and U266B1 cells' comets can be seen in figures 5.2 and 5.3 respectively. Percentage of DNA in the head and tail can be seen to correlate with the brightness and intensity of the comet.

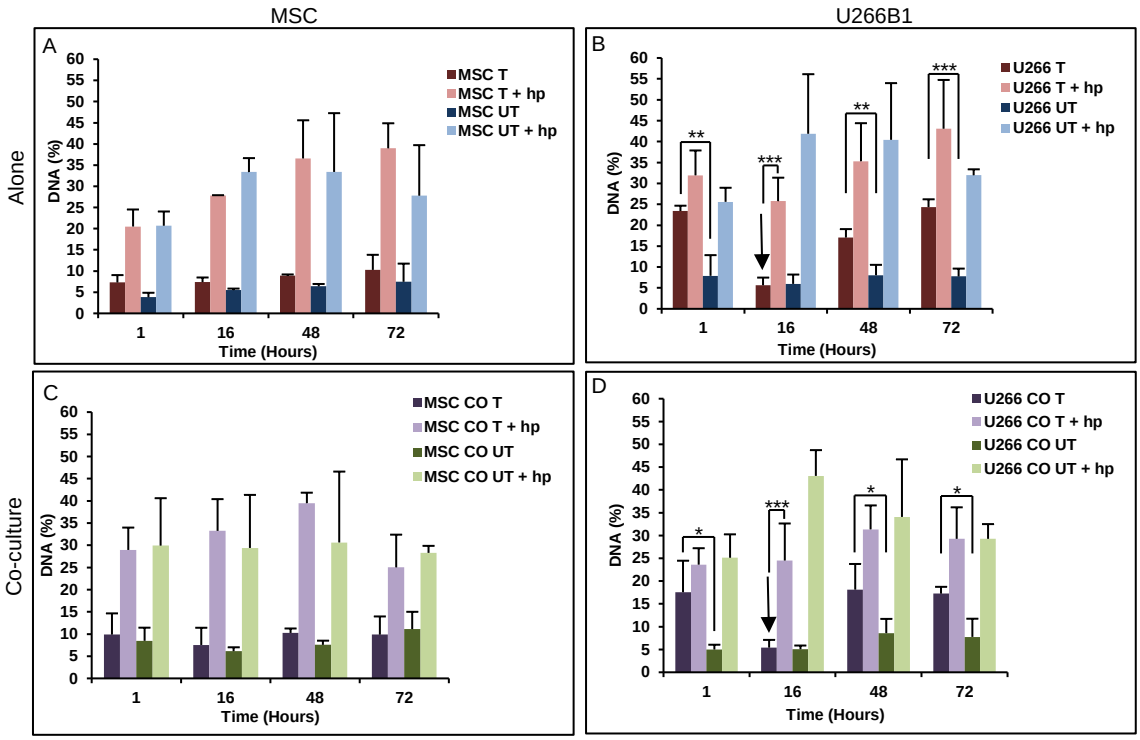

Figure 5.1 DNA damage as measured by comet assay following melphalan exposure. Percentage DNA in the comet tail of MSC (A and C) and U266B1 cells (B and D) after exposure to melphalan (32.8 µM) for 1 hr, either when cultured alone (A and B) or when in co-culture (C and D). Arrows indicate low levels of DNA damage due to comet tail retardation as a result of DNA crosslink formation in U266B1 cells at 16 hrs post exposure. DNA damage was not significantly increased in BM-MSC as a result of melphalan exposure at any time point. Samples taken at 1, 16, 48, 72 hrs post exposure (n=3). A minimum of 50 comets were analysed for each sample. Abbreviations: U266B1 T: U266B1 treated, U266B1 UT: U266B1 untreated, MSC T: Mesenchymal stem cell treated, MSC UT: Mesenchymal stem cell untreated, CO T: U266B1 and MSC treated when cultured together, CO UT: U266B1 and MSC untreated when cultured together, + hp: hydrogen peroxide. Bars represent mean ± SD (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

In both MSC treated and untreated cell samples, small comet tails only, were visible in most cells (figure 5.2). H_2O_2 positive controls indicated high DNA damage at expected levels. Images captured after exposure to H_2O_2, showed a typical comet appearance, confirming that the technique is capable of detecting DNA damage and this corresponds to a very high tail intensity measurement (figure 5.2, F and figure 5.3, F). U266B1 cells had comet tails comparable to that of the untreated control at 16 hrs post exposure to melphalan with increased tail intensities at subsequent time points (figure 5.3).

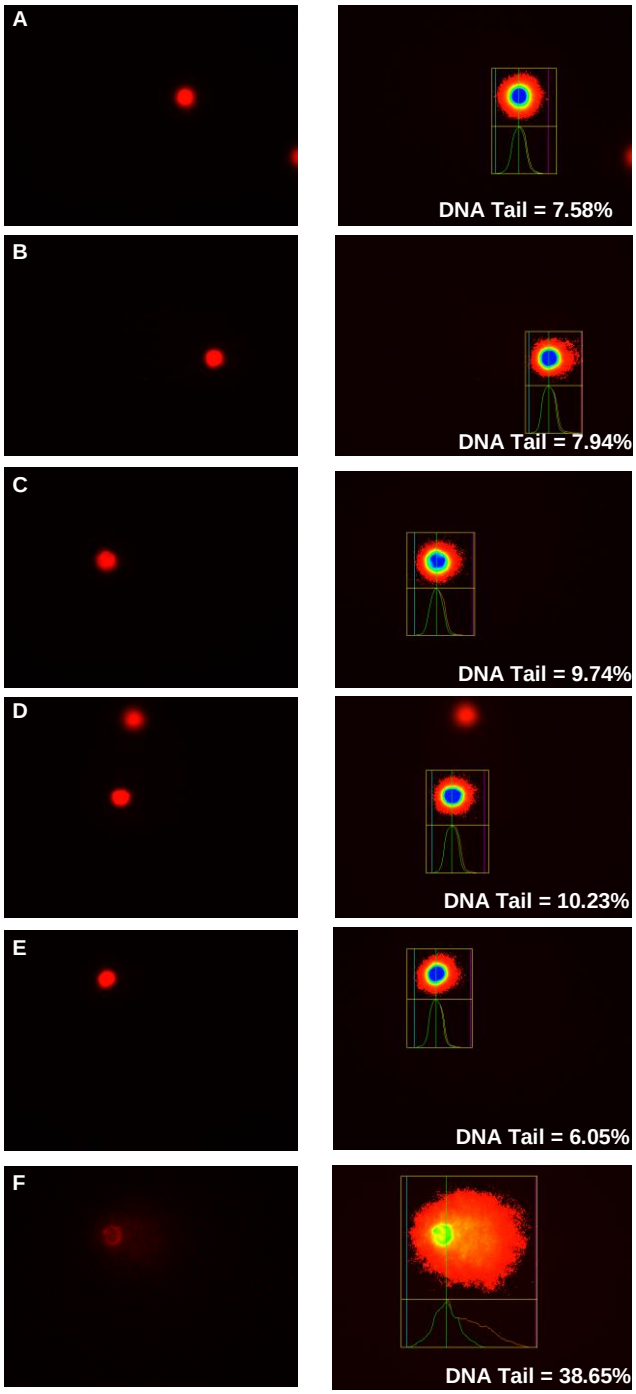

Figure 5.2 Representative images of DNA damage in MSC following melphalan exposure when cultured alone as assessed by alkaline comet assay. Images A – F show various levels of DNA damage in MSC before (left) and after (right) analysis with comet assay IV software. The same cell is depicted in the images on the right following analysis. MSC were exposed to melphalan (32.8 µM) after which they were immediately analysed (A) followed by 16 (B), 48 (C) and 72 hrs (D) post exposure. An untreated sample (E) and a positive control sample (F), utilising H_2O_2 for 5 mins were also performed at each time point. All comet tails intensities, treated and untreated, were relatively small and below 10%. The H_2O_2 control confirms the technique is capable of detecting DNA damage. Untreated sample and positive control images represented here were taken at 48 hrs post exposure. A minimum of 50 comets were analysed for each sample (x 40 magnification).

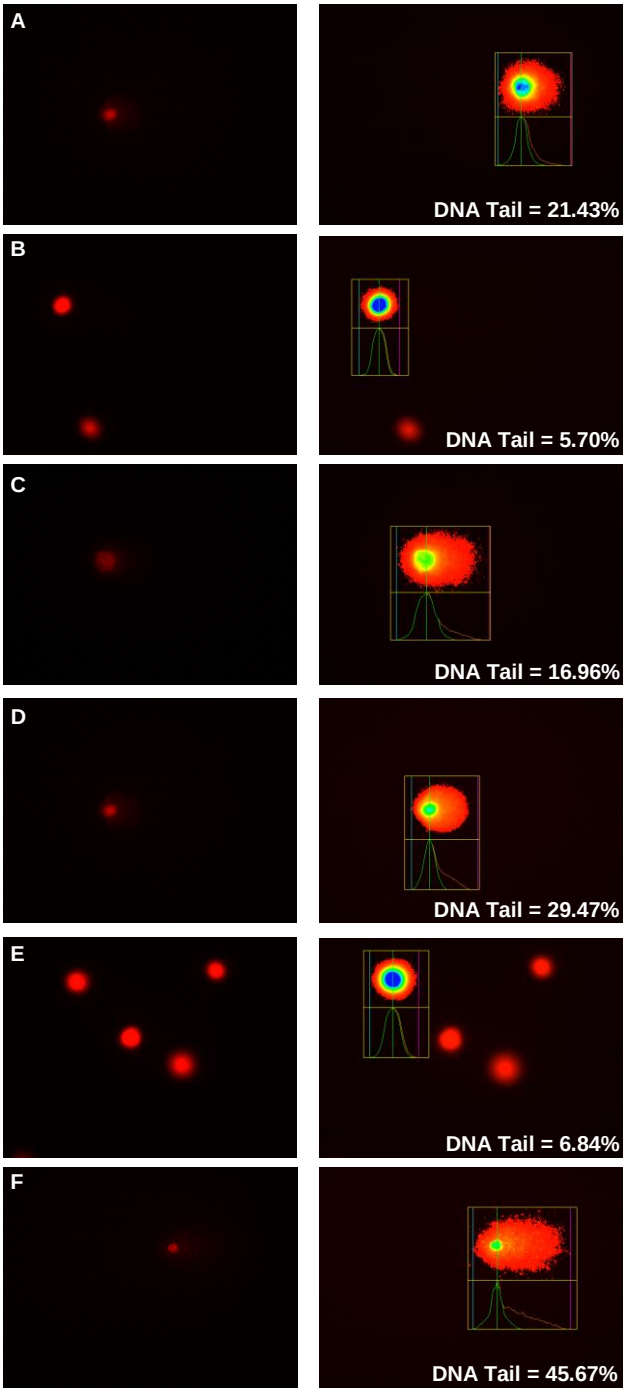

Figure 5.3 Representative images of DNA damage in U266B1 cells following melphalan exposure when cultured alone as assessed by comet assay. Images A – F show various levels of DNA damage in U266B1 cells before (left) and after (right) analysis with comet assay IV software. The same cell is depicted in the images on the right following analysis. U266B1 cells were exposed to melphalan (32.8 µM) after which they were immediately analysed (A) followed by 16 (B), 48 (C) and 72 hrs (D) post exposure. An untreated sample (E) and a positive control sample (F), utilising H_2O_2 for 5 mins were also performed at each time point. Cells that were untreated (E) and those 16 hrs post exposure to melphalan (B) can be seen to have tightly packed DNA with only a small comet tail visible. Untreated sample and positive control images represented here were taken at 48 hrs post exposure. A minimum of 50 comets were analysed for each sample (x 40 magnification).

To investigate a possible bystander effect as a result of melphalan exposure, MSC and U266B1 cells were cultured independently before being exposed to melphalan (32.8 µM) for 1 hr. These treated cells were then removed and co-cultured with previously untreated MSC or U266B1 cells. As illustrated in figure 5.4 there was no significant DNA damage in MSC that were directly exposed to melphalan previously. MSC tail intensities were between 8 – 12% at each time point. MSC that were unexposed to drug and co-cultured with previously treated U266B1 cells had DNA damage levels consistent with the untreated cells until a significant increase in DNA damage at 72 hrs ($p<0.05$). DNA damage in U266B1 cells that had been treated and co-cultured with untreated MSC had reduced crosslink formation at 16 hrs with DNA tail intensity at above 15%. DNA damage in U266B1 at 16 hrs was not significant compared to H_2O_2 controls. DNA tail intensity increased slightly at 48 and 72 hrs being between 15 – 25%. U266B1 cells that were not directly exposed to melphalan and cultured with previously exposed MSC did not produce significant levels of DNA damage at any time point.

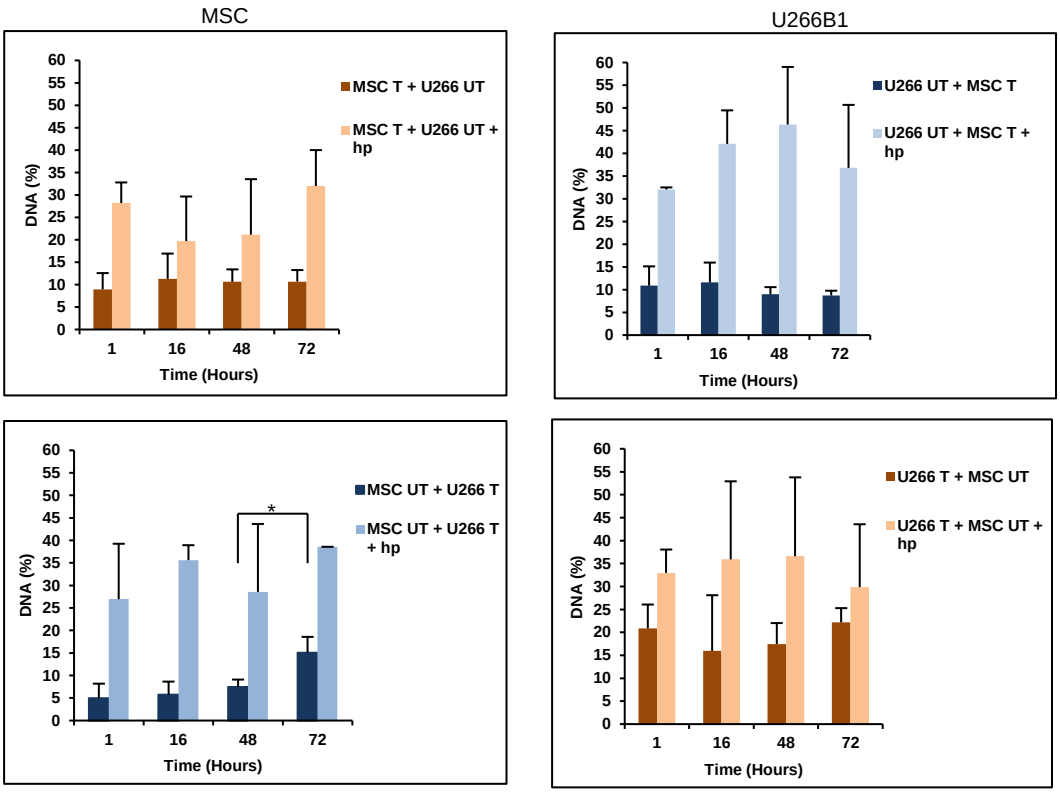

Figure 5.4 DNA damage as measured by comet assay following melphalan exposure either directly or by the exposure of U266B1 or MSC. Percentage DNA in the tail in (A) MSC after exposure to melphalan and co-cultured with untreated U266B1 cells (MSC T + U266B1 UT), (B) untreated U266B1 cells co-cultured with previously melphalan treated MSC (U266B1 UT + MSC T), (C) untreated MSC co-cultured with previously melphalan treated U266B1 cells (MSC UT + U266B1 T), (D) U266B1 cells after exposure to melphalan and co-cultured with untreated MSC (U266B1 T + MSC UT). Melphalan was administered at a dose of 32.8 µM for 1 hr. There was no significant DNA damage in MSC samples directly exposed to melphalan. There was a significant increase in DNA damage in untreated MSC at 72 hrs when cultured with previously treated U266B1 ($p<0.05$). U266B1 cells not directly exposed to melphalan did not have an increase in DNA damage as a result of a co-culture with previously exposed MSC. U266B1 cells directly exposed to melphalan and cultured with untreated MSC had increased DNA damage at 16 hrs as opposed to when cultured alone. Samples taken at 1, 16, 48 and 72 hrs post exposure (n=3). A minimum of 150 comets were analysed for each sample; + hp: hydrogen peroxide. Bars represent mean ± SD (*$p<0.05$).

DNA tail intensities were assessed in both HS5 stromal cells and melphalan resistant U266B1 cells following exposure to melphalan (32.8 µM) (figure 5.5). Levels of DNA damage in HS5 increased at each time point although remained below 10% 72 hrs post exposure to melphalan. HS5 cells co-cultured with U266B1 also did not have significantly high levels of DNA damage as measured by comet assay. Melphalan resistant U266B1 cells had an increase in DNA damage immediately after treatment compared to the control ($p<0.05$). At 16 hrs post exposure levels of DNA damage were reduced and similar to

that of the untreated cells. At 48 ($p<0.05$) and 72 hrs ($p<0.05$) levels of DNA damage continued to increase relative to the control. However levels of DNA damage were lower than that previously seen in U266B1 cells not resistant to melphalan (figure 5.1).

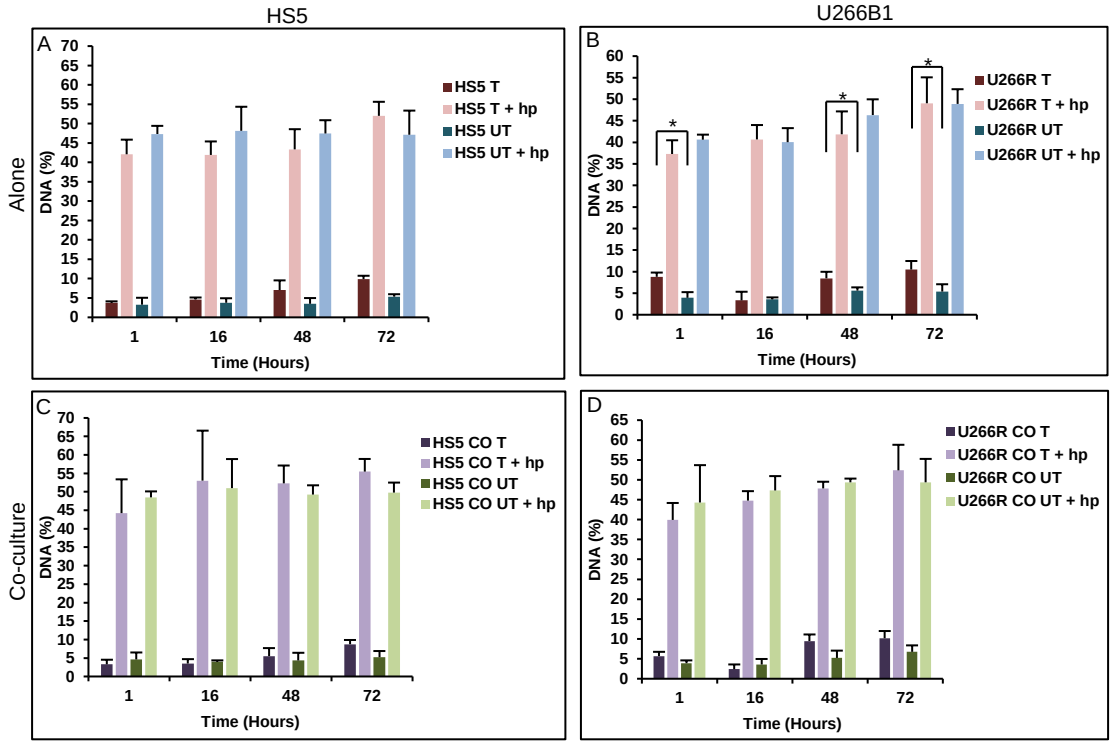

Figure 5.5 DNA damage as measured by comet assay in HS5 stromal cells and melphalan resistant U266B1 cells following melphalan exposure. Percentage DNA in the comet tail of HS5 and melphalan resistant U266B1 cultured alone (A and B) and HS5 and melphalan resistant U266B1 cells when in co-culture(C and D) after exposure to melphalan (32.8 µM) for 1 hr. Samples taken at 1, 16, 48, 72 hrs post exposure (n=3). A minimum of 150 comets were analysed for each sample. Abbreviations: U266B1R T: U266B1 melphalan resistant treated, U266B1R UT: U266B1 melphalan resistant untreated, HS5 T: HS5 treated, HS5 UT: HS5 untreated, CO T: U266B1 melphalan resistant and HS5 treated, CO UT: U266B1 melphalan resistant and HS5 untreated, + hp: hydrogen peroxide. Bars represent mean ± SD (*$p<0.05$).

To investigate a possible bystander effect as a result of melphalan exposure, HS5 and U266B1 resistant cells were cultured independently before being exposed to melphalan (32.8 µM) for 1 hr. These treated cells were then removed and co-cultured with previously untreated HS5 or U266B1 resistant cells. As illustrated in figure 5.6 there was no significant DNA damage in MSC that were directly exposed to melphalan previously.

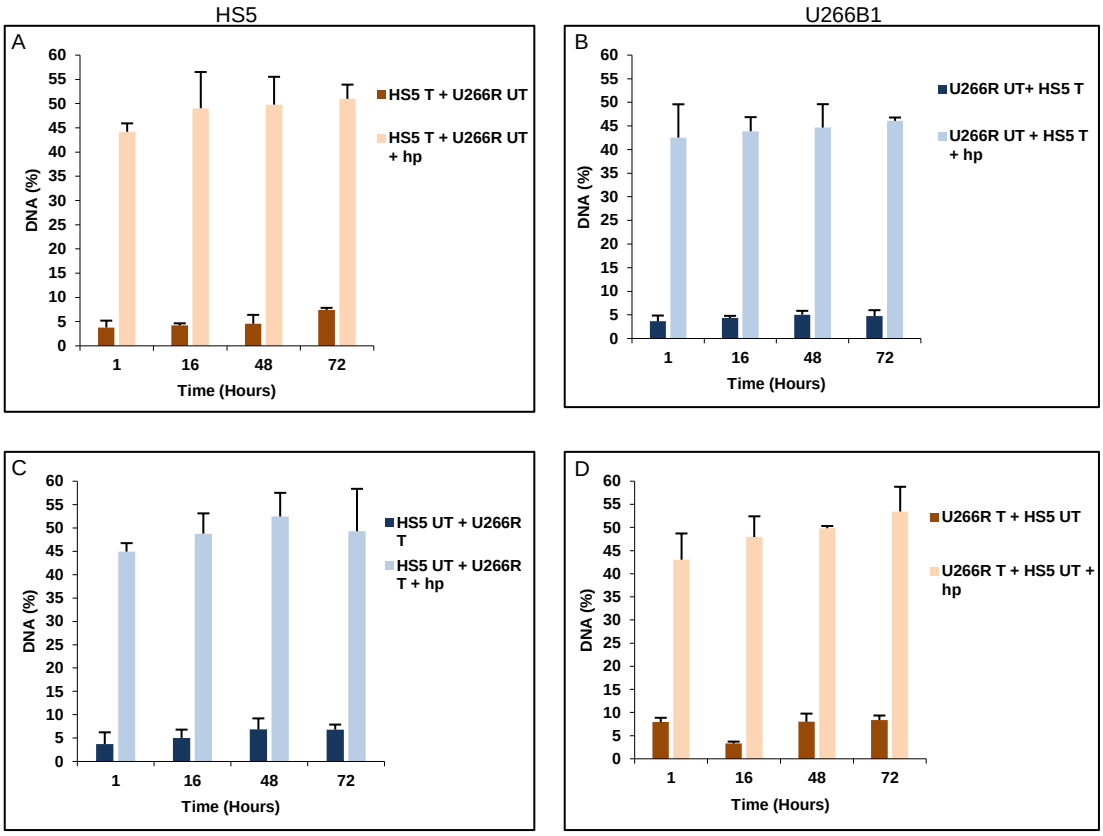

Figure 5.6 DNA damage as measured by comet assay following melphalan exposure either directly or by the indirect exposure of melphalan resistant U266B1 or HS5. Percentage DNA in the tail in (A) untreated HS5 co-cultured with previously melphalan treated melphalan resistant U266B1 cells (HS5 UT + U266B1R T), (B) melphalan resistant U266 cells after exposure to melphalan and co-cultured with untreated (HS5 U266B1R T + HS5 UT), (C) HS5 after exposure to melphalan and co-cultured with untreated melphalan resistant U266B1 cells (HS5 T + U266B1R UT), (D) untreated melphalan resistant U266B1 cells co-cultured with previously melphalan treated HS5 (U266B1R UT + HS5 T). Melphalan was administered at a dose of 32.8 µM for 1 hr. Samples taken at 1, 16, 48 and 72 hrs post exposure (n=3). A minimum of 150 comets were analysed for each sample; + hp: Hydrogen peroxide. Bars represent mean ± SD.

5.3.1.2 Thalidomide

Thalidomide has been shown to be an important therapeutic option for patients with MM as it has a broad range of effects that limit MM cell growth, although its exact mechanism of action still remains unclear (Latif *et al*., 2012). There has been much debate regarding the mutagenicity of thalidomide with conflicting reports expressing that it is non-genotoxic (Teo *et al*., 2000) and others who indicate that it is genotoxic (Huang and McBride, 1997). Here the levels of DNA damage as measured by comet assay induced in U266B1 and HS5 cells following exposure to thalidomide (200 ng / ml) for 1 hr were investigated and compared. Cells were collected immediately (1 hr) as well as at 24, 48 and 72 hrs post exposure.

No significant DNA damage was detected by comet assay, in HS5 and U266B1 independent cultures following thalidomide (200 ng / ml) exposure for 1 hr at any time point (figure 5.7). DNA tail intensities were recorded at levels between 5 and 10% for each of these cultures and were consistant with the untreated controls. When HS5 and U266B1 cells were co-cultured together and exposed to thalidomide, this did not significantly affect the DNA tail intensities in both HS5 and U266B1 cells. DNA damage was below 10% in all treated and untreated HS5 and U266B1 samples follwing a co-culture. H_2O_2 positive controls indicated high DNA damage, with tail intensities above 40% in all independent and co-culture samples.

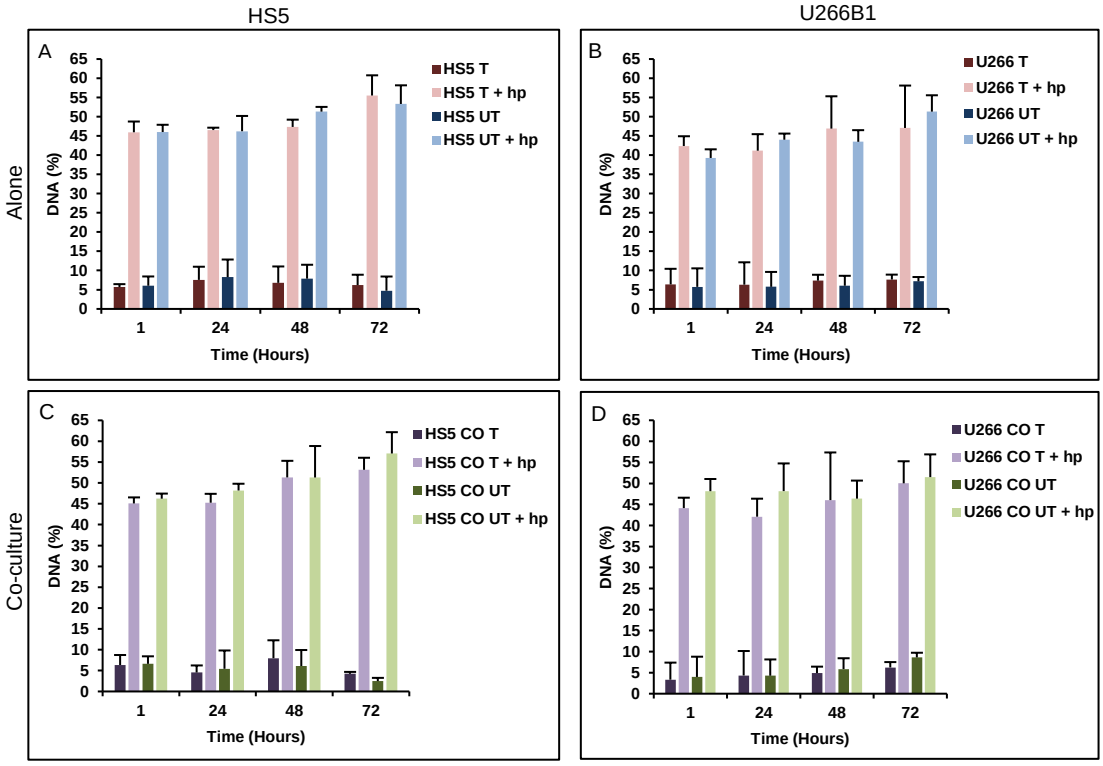

Figure 5.7 DNA damage as measured by comet assay following thalidomide exposure. Percentage DNA in the comet tail in (A) HS5 and (B) U266B1 cells after exposure to thalidomide alone and (C) HS5 and (B) U266B1 cells following a co-culture and after exposure to thalidomide. Thalidomide was administered at a dose of 200 ng / ml for 1 hr. DNA tail intensities following exposure to thalidomide were similar to the untreated in both independent and co-cultures in both cell lines and thus indicates that thalidomide is non-genotoxic according to the comet assay. Samples taken at 1, 24, 48, 72 hrs post exposure (n=3). A minimum of 150 comets were analysed for each sample. Abbreviations: U266B1 T: U266B1 treated, U266B1 UT: U266B1 untreated, HS5 T: HS5 treated, HS5 UT: HS5 untreated, CO T: U266B1 and HS5 treated, CO UT: U266B1 and HS5 untreated, + hp: Hydrogen peroxide. Bars represent mean ± SD.

To investigate a possible bystander effect as a result of thalidomide exposure, HS5 and U266B1 cells were cultured independently before being exposed to thalidomide (200 ng / ml) for 1 hr. Cells were then removed and co-cultured (via an insert) with previously untreated HS5 or U266B1. No significant DNA damage was detected by comet assay, in HS5 and U266B1 following exposure to thalidomide directly or via the indirect exposure of either HS5 or U266B1 cells (figure 5.8). HS5 and U266B1 tail intensities were between 5 – 10% at each time point.

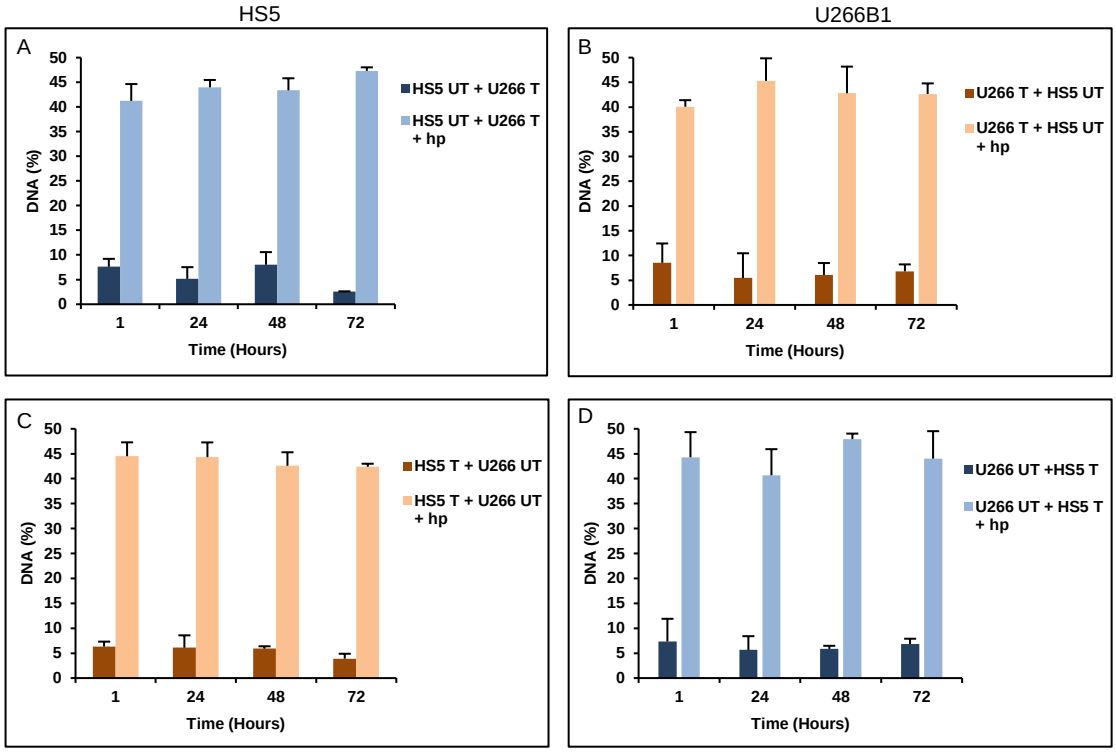

Figure 5.8 DNA damage as measured by comet assay following thalidomide exposure either directly or by the indirect exposure of U266B1 or HS5. Percentage DNA in the tail in (A) untreated HS5 cells co-cultured with previously thalidomide treated U266B1 cells (HS5 UT + U266 T), (B) thalidomide exposed U266B1 cells co-cultured with previously untreated HS5 cells (U266B1 T + HS5 UT), (C) HS5 cells after exposure to thalidomide and co-cultured with untreated U266B1 cells (HS5 T + U266B1 UT), (D) untreated U266B1 cells and co-cultured with thalidomide treated HS5 cells (U266B1 UT + HS5 T). Thalidomide was administered at a dose of 200 ng / ml for 1 hr. No significant DNA damage in HS5 or U266B1 as a result of exposure to previously thalidomide treated cells was evident. Samples taken at 1, 24, 48 and 72 hrs post exposure (n=3). A minimum of 150 comets were analysed for each sample; + hp: Hydrogen peroxide. Bars represent mean ± SD.

5.3.1.3 Lenalidomide

Lenalidomide is a thalidomide derivative with an established efficacy in MM, yet like its parent drug its mechanism of action is not fully understood (Shannon *et al*., 2012). Here the comet assay was used to investigate the potential DNA damage caused by lenalidomide. Levels of DNA damage induced in both U266B1 and HS5 cells following exposure to lenalidomide (4 µM) for 1 hr, were investigated and compared. Cells were collected immediately (1 hr) as well as at 24, 48 and 72 hrs post exposure.

No significant DNA damage was detected by comet assay, in HS5 and U266B1 independent cultures following lenalidomide (4 µM) exposure for 1 hr at any time point (figure 5.9). HS5 and U266B1 cells' DNA tail intensities were between 3 – 5% for all treated and untreated cells. When HS5 and U266B1 cells were co-cultured together and exposed to thalidomide this did not significantly affect the tail intensities and thus the DNA damage in both HS5 and U266B1 cells. DNA damage was below 10% in all treated and untreated HS5 and U266B1 samples. H_2O_2 positive controls increased DNA damage to above 30% in all samples.

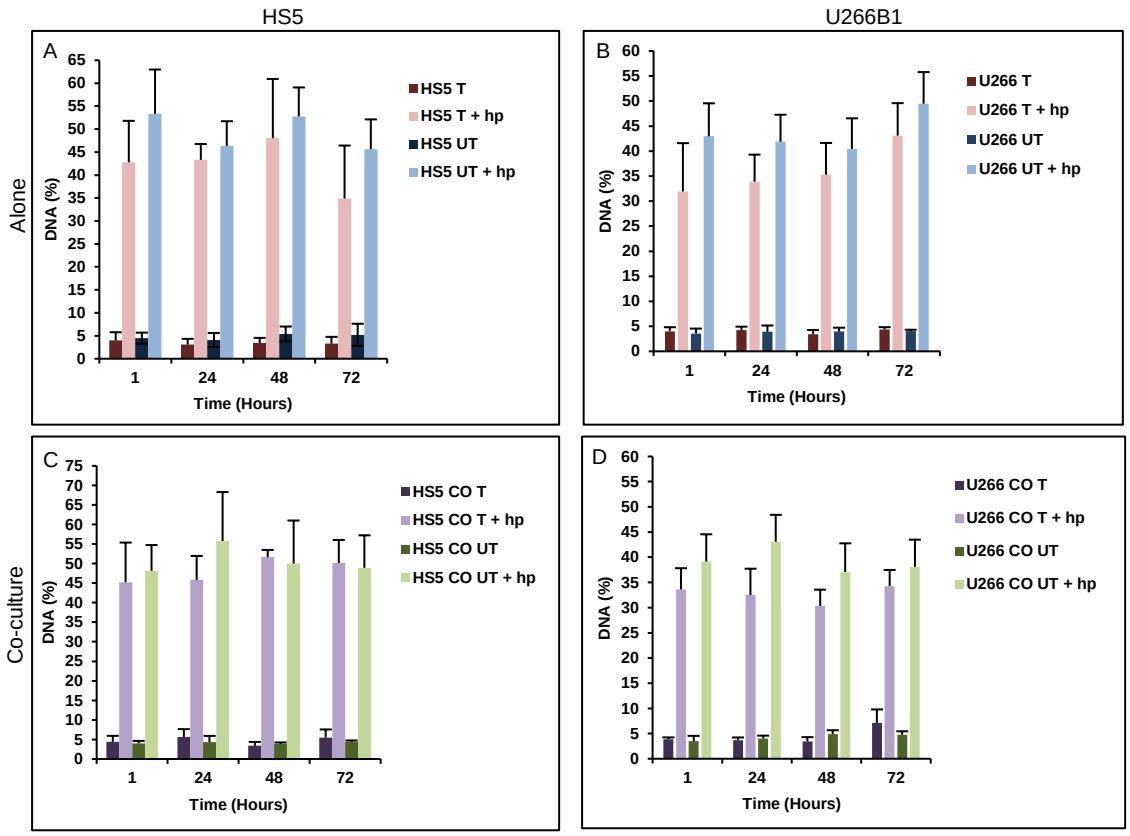

Figure 5.9 DNA damage as measured by comet assay following lenalidomide exposure. Percentage DNA in the comet tail in (A) HS5 and (B) U266B1 cells after exposure to lenalidomide alone (C) HS5 and (B) U266B1 cells following a co-culture and after exposure to lenalidomide. Lenalidomide was administered at a dose of 4 µM for 1 hr. The comet assay shows lenalidomide not to be genotoxic in HS5 and U266B1 cells whether treated independently or as a co-culture. Levels of DNA damage following exposure to lenalidomide were similar to untreated cells in both independent and co-cultures in both cell lines. Samples taken at 1, 24, 48, 72 hrs post exposure (n=3). A minimum of 150 comets were analysed for each sample. Abbreviations: U266B1 T: U266B1 treated, U266B1 UT: U266B1 untreated, HS5 T: HS5 treated, HS5 UT: HS5 untreated, CO T: U266B1 and HS5 treated, CO UT: U266B1 and HS5 untreated, + hp: Hydrogen peroxide. Bars represent mean ± SD.

To investigate a possible bystander effect as a result of lenalidomide exposure, HS5 and U266B1 cells were cultured independently and exposed to lenalidomide (4 µM) for 1 hr. Cells were then washed free of drug and co-cultured (via an insert) with previously unexposed HS5 or U266B1.

U266B1 and HS5 cells exposed indirectly to lenalidomide via the previous exposure of opposing cells in a co-culture indicated no significant DNA damage (figure 5.10). DNA damage as measured by DNA tail intensity was

below 10% in all samples. Positive controls with H_2O_2 again showed consistency with DNA damage in these samples above 30%.

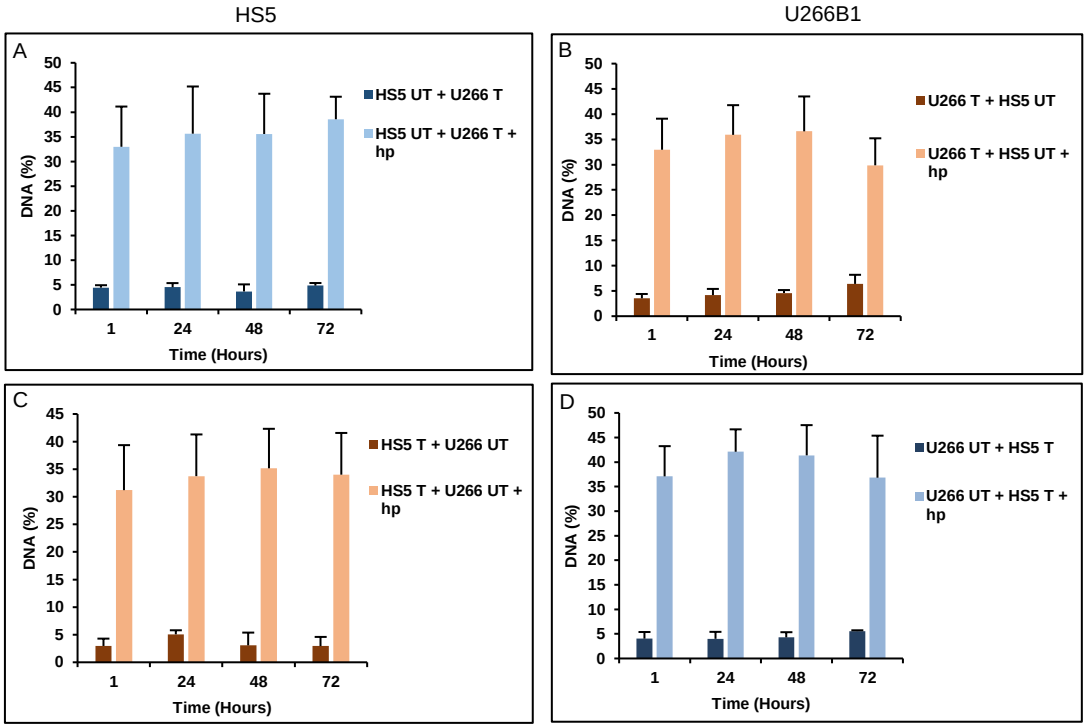

Figure 5.10 DNA damage as measured by comet assay following lenalidomide exposure either directly or by the exposure of U266B1 or HS5. Percentage DNA in the tail in (A) untreated HS5 cells co-cultured with previously treated U266B1 cells (U266B1 T + HS5 UT), (B) U266B1 cells after exposure to lenalidomide and co-cultured with untreated HS5 cells (U266B1 UT + HS5 T), (C) HS5 cells after exposure to lenalidomide (4 µM) and co-cultured with untreated U266B1 cells (HS5 T + U266B1 UT), (D) untreated U266B1 cells co-cultured with previously treated HS5 cells (HS5 UT + U266B1 T). Lenalidomide was administered at a dose of 4 µM for 1 hr. No significant levels of DNA damage can be seen in either cell type either exposed directly or cultured with a previously exposed cell line. Therefore no evidence of a bystander effect was reported in both U266B1 and HS5 cells. Samples taken at 1, 24, 48 and 72 hrs post exposure (n=3). A minimum of 150 comets were analysed for each sample; + hp: Hydrogen peroxide. Bars represent mean ± SD.

5.3.1.4 Bortezomib

Bortezomib has been shown to impair HR repair of DNA breaks in MM cells (Neri *et al*., 2011). Here levels of DNA damage in both U266 and HS5 cells following exposure to bortezomib (500 nM) for 1 hr were investigated and compared. Cells were collected immediately (1 hr) as well as at 24, 48 and 72 hrs post exposure.

No significant DNA damage was detected by comet assay, in HS5 and U266B1 independent cultures following bortezomib (500 nM) exposure for 1 hr at any time point (figure 5.11). DNA tail intensities were between 3 - 5% in HS5 cells and between 5 – 10% in U266B1 cells at each time point following bortezomib exposure. When HS5 and U266B1 cells were co-cultured together and exposed to bortezomib this did not significantly affect the tail intensities of these cells and thus the DNA damage in both HS5 and U266B1 cells remained at control levels. DNA damage was between 5 - 10% in all treated and untreated HS5 and U266B1 co-cultured samples. H_2O_2 positive controls indicated high DNA damage at levels above 30% in all samples.

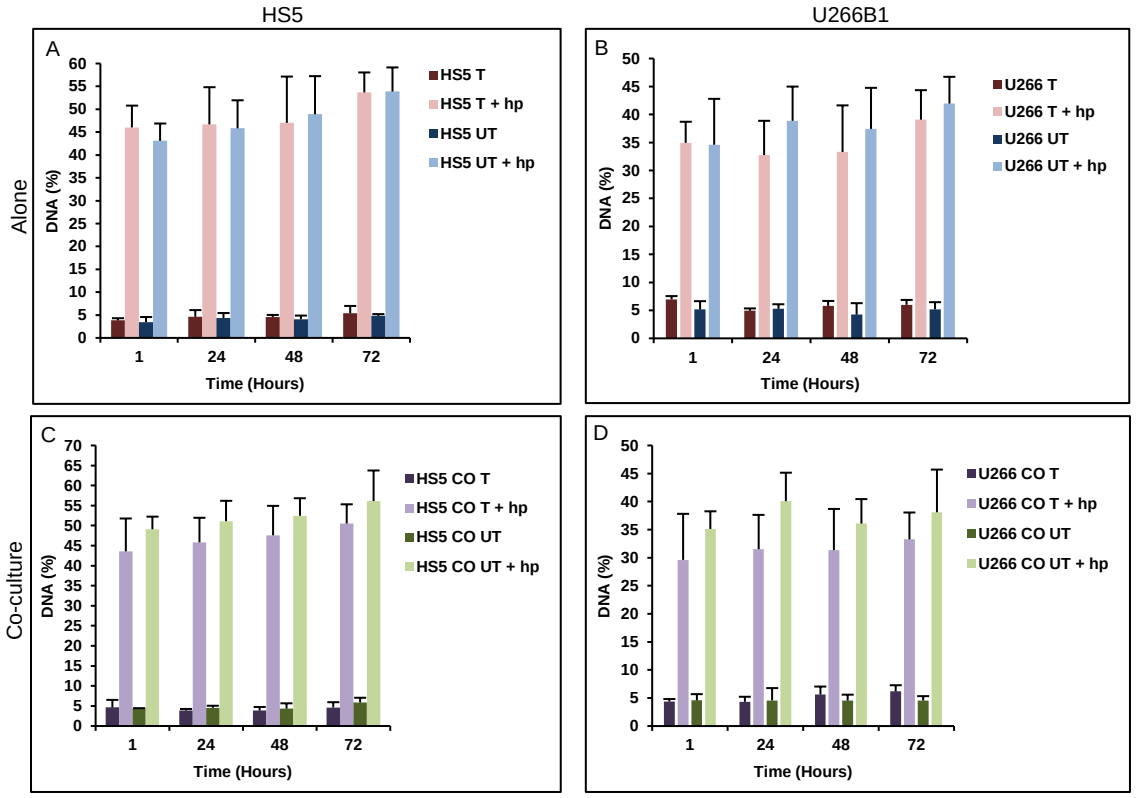

Figure 5.11 DNA damage as measured by comet assay following bortezomib exposure. Percentage DNA in the comet tail of HS5 (A and C) and U266B1 cells (B and D) after exposure to bortezomib (500 nM) either alone (A and B) and when in co-culture (C and D). Bortezomib exposure does not increase DNA damage in U266B1 and HS5 cells as measured by comet assay. Samples were taken at 1, 24, 48, 72 hrs post exposure (n=3). A minimum of 150 comets were analysed for each sample. Abbreviations: U266B1 T: U266B1 treated; U266B1 UT: U266B1 untreated; HS5 T: HS5 treated; HS5 UT: HS5 untreated; CO T: U266B1 and HS5 treated; CO UT: U266B1 and HS5 untreated; + hp: Hydrogen peroxide. Bars represent mean ± SD.

To investigate a possible bystander effect as a result of bortezomib exposure, HS5 and U266B1 cells were initially cultured independently and exposed to bortezomib (500 nM) for 1 hr. Cells were then removed and co-cultured (via an insert) with previously untreated HS5 or U266B1.

U266B1 and HS5 cells exposed indirectly to bortezomib via the previous exposure of opposing cells in a co-culture indicated no significant DNA damage (figure 5.12). DNA damage as measured by DNA tail intensity was below 10% in all samples. Positive controls with H_2O_2 again showed consistency with DNA damage in these samples above 30%.

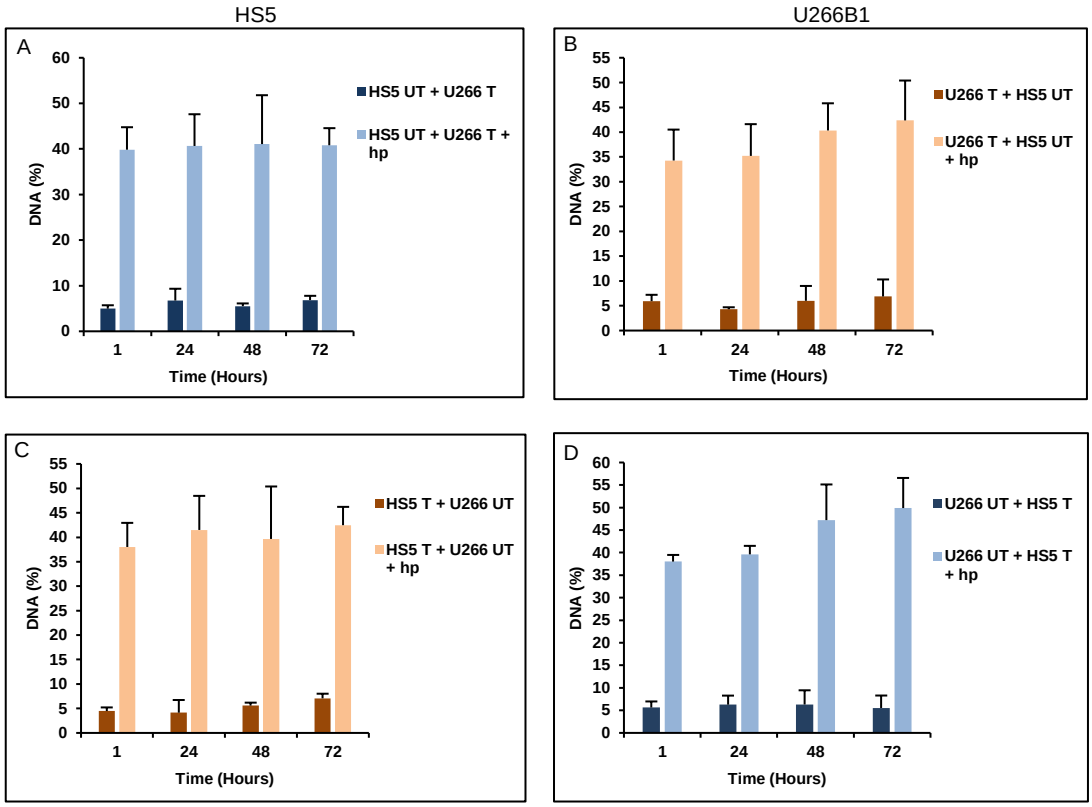

Figure 5.12 DNA damage as measured by comet assay following bortezomib exposure either directly or by the indirect exposure of U266B1 or HS5. Percentage DNA in the tail in (A) untreated HS5 cells co-cultured with previously treated U266B1 cells (U266B1 UT + HS5 T), (B) U266B1 cells after exposure to bortezomib and co-cultured with untreated HS5 cells (U266B1 T + HS5 UT), (C) HS5 cells after exposure to bortezomib and co-cultured with untreated U266B1 cells (HS5 T + U266B1 UT), (D) untreated U266B1 cells co-cultured with previously treated HS5 cells (HS5 UT + U266B1 T). Levels of DNA damage in HS5 and U266B1 cells were consistent with independent untreated controls with no significant increase in DNA damage. As such no bystander effect appears to be present following bortezomib exposure when analysed with the comet assay. Samples taken at 1, 24, 48 and 72 hrs post exposure (n=3). A minimum of 150 comets were analysed for each sample; + hp: Hydrogen peroxide. Bars represent mean ± SD.

5.3.1.5 Carfilzomib

The levels of DNA damage induced in both U266B1 and HS5 cells following exposure to carfilzomib (13.8 nM) for 1 hr were investigated and compared. Cells were collected immediately (1 hr) as well as at 24, 48 and 72 hrs post exposure.

No significant levels of DNA damage were detected by comet assay, in HS5 and U266B1 independent cultures following carfilzomib (13.8 nM) exposure for 1 hr at any time point (figure 5.13). When HS5 and U266B1 cells were co-cultured togther and exposed to carfilzomib this did not significantly affect the tail intensities and thus the DNA damage in both HS5 and U266B1 cells. DNA damage was below 10% in all treated and untreated HS5 and U266B1 samples. H_2O_2 positive controls indicated high DNA damage at expected levels.

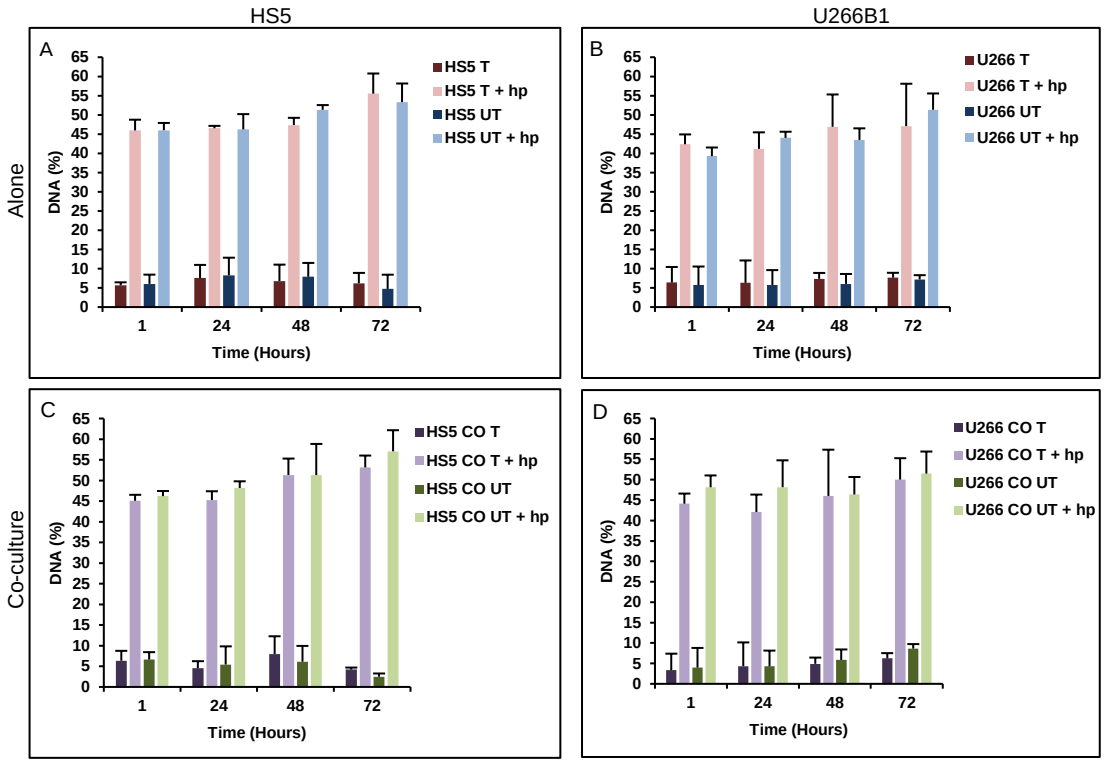

Figure 5.13 DNA damage as measured by comet assay following carfilzomib exposure. Percentage DNA in the comet tail of HS5 (A and C) and U266B1 cells (B and D) after exposure to carfilzomib (13.8 nM) either alone (A and B) and when in co-culture (C and D). No increase in DNA damage was recorded by the comet assay as a result of carfilzomib treatment to U266B1 and HS5 cells independently and when in co-culture. Samples taken at 1, 24, 48, 72 hrs post exposure (n=3). A minimum of 150 comets were analysed for each sample. Abbreviations: U266B1 T: U266B1 treated; U266B1 UT: U266B1 untreated; HS5 T: HS5 treated; HS5 UT: HS5 untreated; CO T: U266B1 and HS5 treated; CO UT: U266B1 and HS5 untreated; + hp: Hydrogen peroxide. Bars represent mean ± SD.

To investigate a possible bystander effect as a result of carfilzomib (13.8 nM) exposure, HS5 and U266B1 cells were cultured independently before being exposed to carfilzomib (13.8 nM) for 1 hr. Cells were then washed free of drug and co-cultured (via an insert) with previously unexposed HS5 or U266B1.

U266B1 and HS5 cells exposed indirectly to carfilzomib via the previous treatment of opposing cells in a co-culture indicated no significant DNA damage (figure 5.14). DNA damage as measured by DNA tail intensity was below 10% in all samples. Positive controls with H_2O_2 again showed consistency with DNA damage in these samples above 30%.

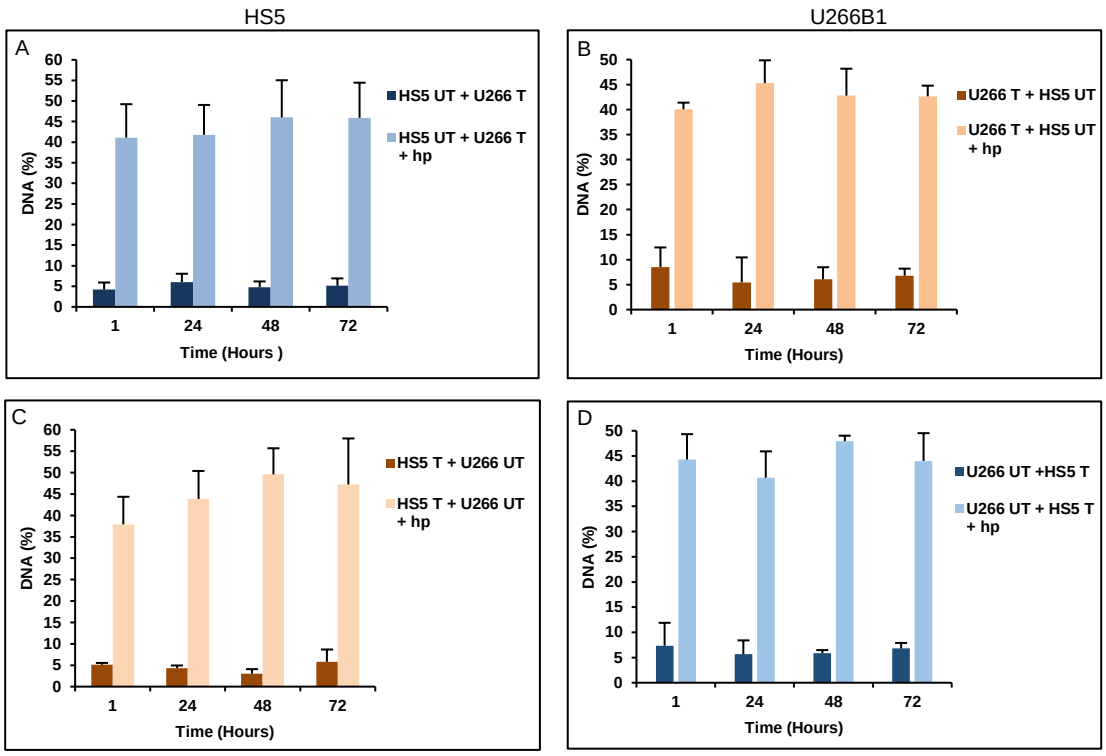

Figure 5.14 DNA damage as measured by comet assay following carfilzomib exposure either directly or by the indirect exposure of U266B1 or HS5. Percentage DNA in the tail in (A) untreated HS5 cells co-cultured with previously treated U266B1 cells (HS5 UT + U266B1 T), (B) U266B1 cells after exposure to carfilzomib and co-cultured with untreated HS5 cells (U266B1 T + HS5 UT), (C) HS5 cells after exposure to carfilzomib (13.8 nM) and co-cultured with untreated U266B1 cells (HS5 T + U266B1 UT), (D) untreated U266B1 cells co-cultured with previously treated HS5 cells (U266B1 UT + HS5 T). No significant levels of DNA damage can be seen in either cell type either exposed directly or cultured with a previously exposed cell line. Therefore no evidence of a bystander effect was reported in both U266B1 and HS5 cells. Samples taken at 1, 24, 48 and 72 hrs post exposure (n=3). A minimum of 150 comets were analysed for each sample; + hp: Hydrogen peroxide. Bars represent mean ± SD.

5.3.2 Micronucleus Assay Results

The micronucleus assay is a test widely used, both *in vitro* and *in vivo*, to assess the genotoxicity of chemicals and the consequent risk to humans. The assay when performed appropriately detects both clastogenicity (chromosome breakage) and aneugenicity (chromosome loss due to dysfunction of mitotic apparatus) (Araldi *et al*., 2015). Micronuclei are DNA fragments or whole chromosomes that are separated from the main nucleus during cell division and are an indication of genotoxicity (Sabharwal *et al*., 2015). The *in vitro* micronucleus test is an umbrella term for many different micronucleus tests such as those with and without cytochalasin-B.

5.3.2.1 Cytochalasin-B treatment of U266B1 cells

The cytokinesis-block micronucleus assay specifically restricts scoring of cells to those that have completed one cell division, and are referred to as bi-nucleate (Zelazna *et al*., 2011). Cytochalasin-B is an inhibitor of the microfilament ring assembly required for the completion of cytokinesis (Trendowski, 2015). In this study cytochalasin-B was initially used in U266B1 cells following treatment of cells with melphalan (32.8 µM). Cells were to be scored for micronuclei 24 hrs following cytochalasin-B exposure. However, upon imaging of cells it was clear that the cytochalasin-B was toxic to the U266B1 cells with a very limited number of complete cells visible (figure 5.15).

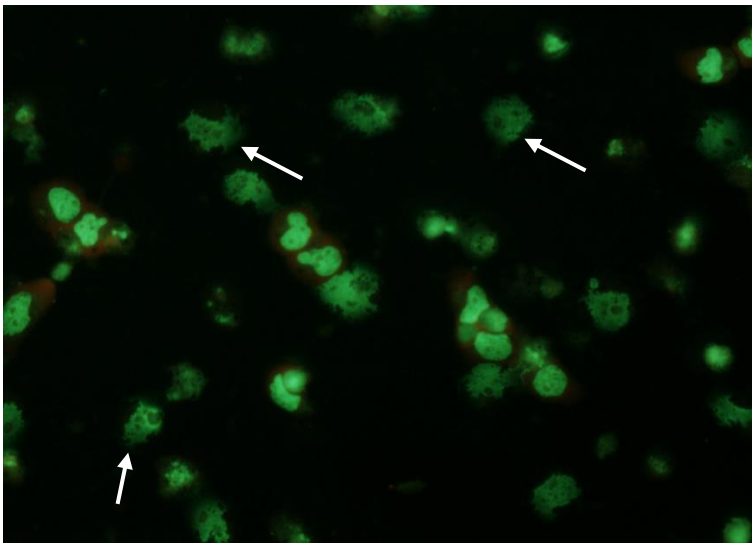

Figure 5.15 Representative image of U266B1 cells following exposure to cytochalasin-B. Cells lacked a clearly defined nucleus and cytoplasm and appeared necrotic (arrowed) (n=3) (x 40 magnification).

5.3.2.2 Non cytochalasin-B micronucleus assay

As cytochalasin-B exposure of U266B1 cells failed to capture cells following one cell division successfully, a non- cytochalasin-B method was used. OECD test guidelines also allow the use of protocols without cytokinesis block, provided there is evidence that the majority of cells analysed are likely to have undergone cell division. As recommended by OECD guidelines, the relative increase in cell count (RICC), was calculated in order to estimate the cytotoxicity of each treatment. The equation used to calculate the RICC can be found in section 2.5.1.4. For regulatory testing of pharmaceuticals RICC values should not be below 50% (± 5%). However for the purposes of this thesis samples with an RICC below 50% (± 5%) have been retained.

The non-cytochalasin-B micronucleus assay was used to assess levels of DNA damage following exposure to melphalan, thalidomide, lenalidomide, bortezomib and carfilzomib for 1 hr at their clinical doses. At several time points after drug exposure cells were harvested and analysed with the micronucleus assay. Each sample was manually scored for micro-nucleated (MN), bi-nucleated (BN), and multi-nucleated cells.

Data was analysed using excel software with the mean of three independent experiments used to create the graphs.

Primary MSC and HS5 stromal cells were exposed to melphalan (32.8 µM) for 1 hr independently and measured for MN at 1, 24, 48 and 72 hrs post exposure. Primary MSC were also co-cultured with U266B1 cells via an insert and exposed to melphalan. HS5 stromal cells were co-cultured via an insert with melphalan resistant U266B1 cells. RICC were calculated for all MSC and HS5 stromal cultures exposed to melphalan (table 5.1).

MSC exposed to melphalan (32.8 µM) for 1 hr when cultured independently gave rise to an increase in MN over a 72 hr period (figure 5.16). MSC had significant numbers of MN compared to the control at 24 ($p<0.01$), 48 ($p<0.05$) and 72 hrs ($p<0.01$) post exposure. Numbers of MN also increased when in co-culture with U266B1 cells, although numbers of MN were reduced compared to independent cultures. The median numbers of MN in MSC cultured independently at 72 hrs was 13.3 compared to 5.3 when co-cultured with U266B1 suggesting protection of MSC by U266B1 cells.

HS5 cells also had an increase in MN over a 72 hr period when cultured independently and exposed to melphalan (32.8 µM) (figure 5.16). MN were significantly higher at 24 ($p<0.01$), 48 ($p<0.01$) and 72 hrs ($p<0.01$) post exposure to melphalan. HS5 cells that were co-cultured with melphalan resistant U266B1 cells also had an increase in MN. However numbers of MN were again reduced when in co-culture compared to alone cultures. Numbers of MN were significantly higher at 24 ($p<0.05$), 48 ($p<0.05$) and 72 hrs ($p<0.001$) compared to the control.

Time (Hours)	**Relative Increase in Cell Counts (RICC) (%)**			
	MSC	**HS5**	**MSC**	**HS5**
	Alone	**Alone**	**Co-culture**	**Co-culture**
1	60.0	80	45	84
16	69.0	64	84	89
48	46.0	67	51	50
72	67.0	53	78	45

Table 5.1 RICC of MSC and HS5 cells cultured independently or in co-culture with U266B1 sensitive or melphalan resistant cells. RICC were greater than 50% (± 5%) at each time point for MSC and HS5.

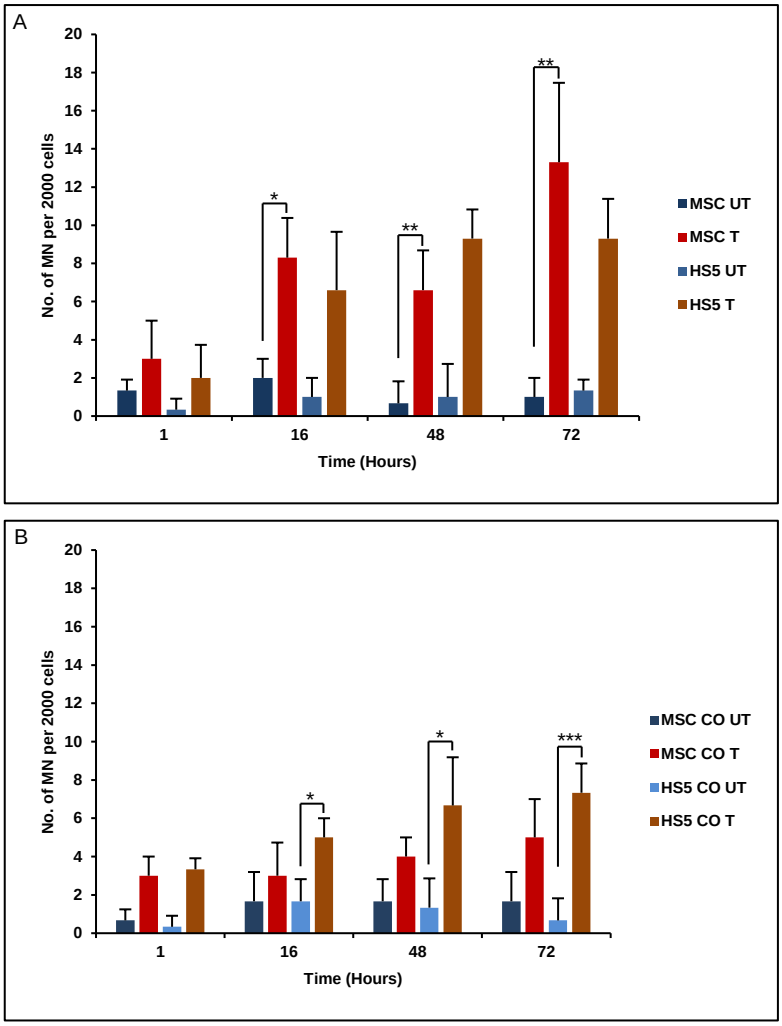

Figure 5.16 Numbers of micronuclei (MN) in primary MSC and HS5 stromal cells over a 72 hr period following exposure to Melphalan. Primary MSC and HS5 cells were exposed to melphalan (32.8 µM) for 1 hr either alone (A) or in co-culture with U266B1 (B) (primary MSC were co-cultured with U266B1 melphalan sensitive cells whereas HS5 were co-cultured U266B1 melphalan resistant cells). Number of MN in MSC and HS5 increased at each time point compared to the control when cultured independently. The numbers of MN in MSC and HS5 were lower when in co-culture with their respective U266B1 compared to independent culture. Samples taken at 1, 24, 48 and 72 hrs post exposure (n=3). Abbreviations: MSC T, mesenchymal stem cell treated; MSC UT, mesenchymal stem cell untreated; HS5 T, HS5 stromal cell treated; HS5 UT, HS5 stromal cell untreated; MSC CO T, mesenchymal stem cell treated co-cultured with U266B1 treated; MSC CO UT, mesenchymal stem cell co-cultured with U266B1 untreated; HS5 CO T, HS5 stromal cells co-cultured with melphalan resistant U266B1 treated; HS5 CO UT, HS5 stromal cells co-cultured with melphalan resisistant U266B1 untreated. Bars represent mean ± SD (*p<0.05, ** p<0.01, *** p<0.001).

U266B1 sensitive cells and melphalan resistant U266B1 cells were exposed to melphalan or left untreated when cultured independently and while in co-culture with primary MSC or HS5 stromal cells (primary MSC were co-cultured with U266B1 melphalan sensitive cells whereas HS5 were co-cultured U266B1 melphalan resistant cells). RICC were calculated for U266B1 sensitive and melphalan resistant cells cultured alone and in co-culture with MSC/HS5 (table 5.2).

The number of MN in U266B1 cells increased in a time dependant manner compared to the untreated control (figure 5.17). U266B1 cells had significantly higher numbers of MN at 24 ($p<0.05$), 48 ($p<0.01$) and 72 hrs ($p<0.01$) post exposure to melphalan. Melphalan resistant U266B1 cells did not have a significant increase in MN until 72 hrs compared to the control ($p<0.01$).

Numbers of MN were reduced in U266B1 cells co-cultured with MSC compared to independent cultures (figure 5.17, B). MN were significantly higher in U266B1 cells co-cultured with MSC at 48 hrs compared to the control ($p<0.01$). Numbers of MN reduced at 72 hrs but still remained significantly higher than the control ($p<0.01$). However the median number of MN in U266B1 cells at 48 hrs was 7.6 in co-culture compared to 12 in independent cultures at 48 hrs post exposure. Melphalan resistant U266B1 cells had numbers of MN similar to that of the control following exposure to melphalan when in co-culture with HS5 cells. Together these findings suggest that MSC and HS5 protect sensitive and melphalan resistant U266B1 cells from genotoxic damage following melphalan exposure.

Time (Hours)	Relative Increase in Cell Counts (RICC) (%)			
	U266S	U266R	U266S	U266R
	Alone	Alone	Co-culture	Co-culture
1	58	77	30	56
24	11	58	33	52
48	6	50	14	56
72	1.3	45	35	66

Table 5.2 RICC of U266B1 sensitive and U266B1 melphalan resistant cells cultured independently or in co-culture with either MSC or HS5. Melphalan sensitive cells had an RICC below 50% at 16, 48 and 72 hrs post exposure when cultured alone. When cultured with MSC RICC was below 50% at each time point. Melphalan resistant U266B1 cells had RICC values greater than 50% (± 5%) at each time point

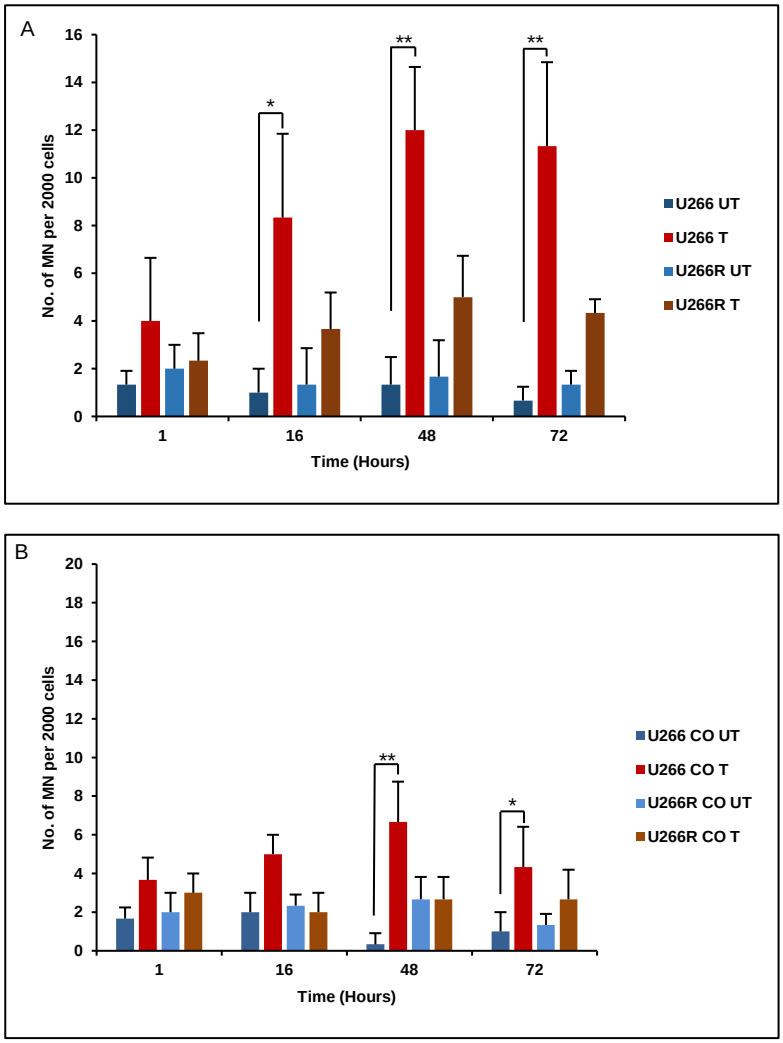

Figure 5.17 Numbers of micronuclei (MN) in U266B1 sensitive and melphalan resistant cells over a 72 hr period following exposure to Melphalan. U266B1 and U266B1 melphalan resistant cells were exposed to melphalan (32.8 µM) for 1 hr either alone (A) or in co-culture with primary MSC/HS5 (B). (Primary MSC were co-cultured with U266B1 melphalan sensitive cells whereas HS5 were co-cultured with U266B1 melphalan resistant cells). Numbers of MN in U266B1 sensitive cells increased at each time point following exposure to melphalan compared to the control when cultured independently. Numbers of MN are reduced following a co-culture with either MSC or HS5. U266B1 melphalan resistant cells have a small increase in MN when cultured independently compared to control. Numbers of MN in U266B1R cells are similar to that of the control when in co-culture with HS5. Samples taken at 1, 24, 48 and 72 hrs post exposure (n=3). Abbreviations: U266B1 T, U266B1 treated; U266B1 UT, U266B1 untreated; U266B1R UT, U266B1 melphalan resistant untreated; U266B1R T, U266B1 melphalan resistant treated; U266B1 CO T U266B1 cells co-cultured with MSC and treated; U266B1 CO UT, U266B1 cells co-cultured with MSC untreated; U266B1R CO T, melphalan resistant U266B1 cells co-cultured with HS5 and treated; U266B1R CO UT, melphalan resistant U266B1 cells co-cultured with HS5 and untreated. Bars represent mean ± SD (*$p<0.05$, ** $p<0.01$).

HS5 and U266B1 cells were cultured independently and co-cultured together via an insert and exposed to either thalidomide (200 ng / ml), lenalidomide (4 µM), bortezomib (500 nM), carfilzomib (13.8 nM) or left untreated. RICC calculated for HS5 cells cultured alone or in co-culture with U266B1 cells can be seen in table 5.3. Cells were measured for MN at 1, 24, 48 and 72 hrs post exposure. Images of MN in HS5 cells following exposure to thalidomide can be seen in figure 5.18.

Time (Hours)	Relative Increase in Cell Counts (RICC) (%)							
	Thalidomide	Lenalidomide	Bortezomib	Carfilzomib	Thalidomide	Lenalidomide	Bortezomib	Carfilzomib
	Alone	Alone	Alone	Alone	Co-culture	Co-culture	Co-culture	Co-culture
1	75	98	98	97	88	93	73	98
24	98	66	76	80	89	98	86	99
48	84	62	45	49	66	89	17	67
72	83	50	9	28	48	72	14	64

Table 5.3 RICC of HS5 cells either cultured independently or in co-culture with U266B1 cells. HS5 cells exposed to bortezomib or carfilzomib had a RICC below 50% at 72 hrs when cultured independently and at 72 hrs when co-cultured with U266 and exposed to bortezomib.

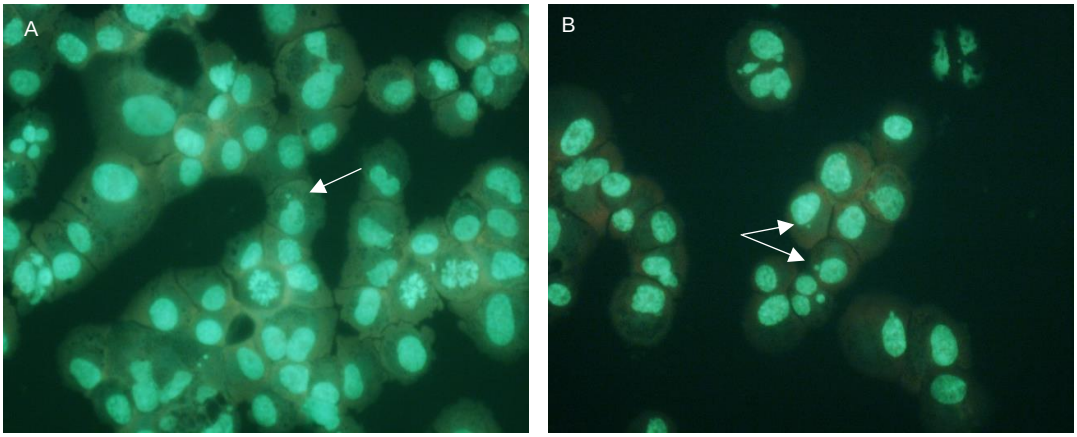

Figure 5.18 Representative images of micronuclei in HS5 cells following exposure to chemotherapy. HS5 and U266B1 cells were cultured independently and co-cultured together and exposed to thalidomide (200 ng / ml) for 1 hr. Cells were harvested and stained with acridine orange. Numbers of micronuclei per 2000 mono-nuclear were scored. A) HS5 cells cultured independently and left untreated at 24 hrs, B) HS5 cells cultured independently and exposed to thalidomide, 48 hrs post exposure (x 40 magnification).

Numbers of MN in HS5 cells increased in a time dependent manner following exposure to thalidomide (figure 5.19). However MN were not significantly higher post exposure compared to the untreated control ($p>0.05$) at any time point. Lenalidomide exposure increased numbers of MN at each time point. Although none were significantly different to the control ($p>0.05$). Proteasome inhibitors bortezomib and carfilzomib gave rise to MN at similar levels to the control.

MN in HS5 cells that were in co-culture with U266B1 reduced compared to independent cultures following exposure to thalidomide. At 72 hrs the average number of MN was 6.3 in independent culture compared to 5 in co-culture when exposed to thalidomide. Numbers of MN in co-cultures exposed to lenalidomide remained at similar levels to when in alone culture. Numbers of MN following exposure to bortezomib and carfilzomib again remained at a level consistent with that of the control.

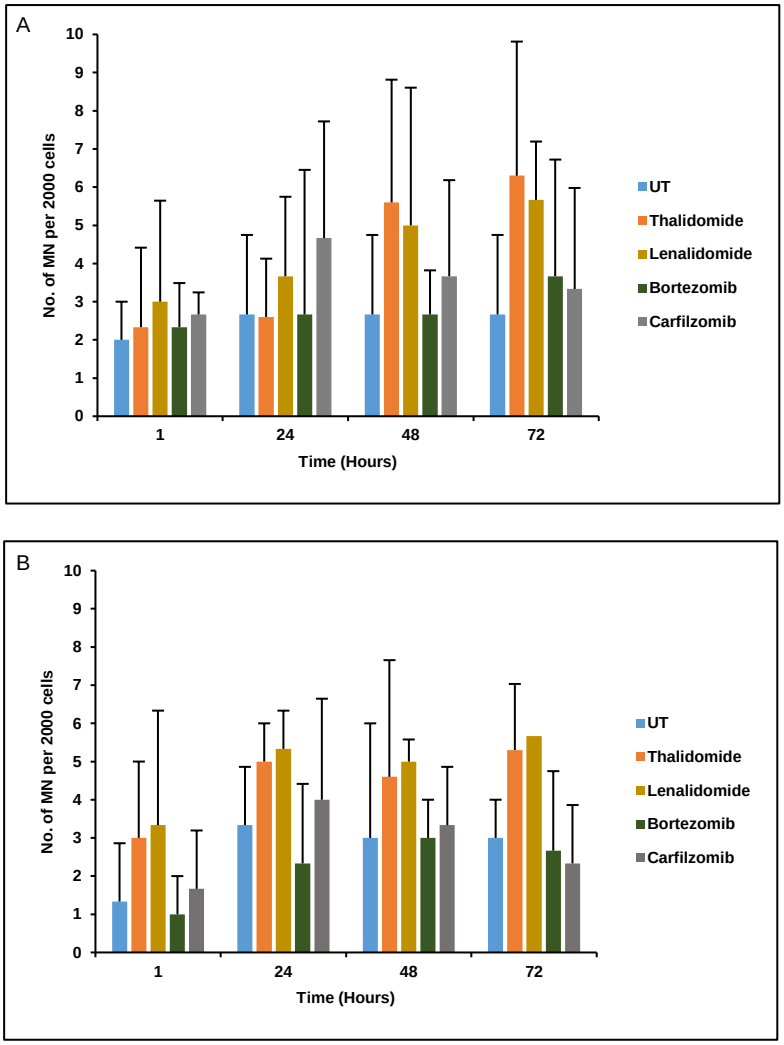

Figure 5.19 Numbers of micronuclei (MN) in HS5 stromal cells over a 72 hr period following exposure to chemotherapy. HS5 cells were exposed to either thalidomide (200 ng / ml), lenalidomide (4 µM), bortezomib (500 nM), carfilzomib (13.8 nM) or left untreated. HS5 stromal cells were exposed to each chemotherapeutic agent for 1 hr either alone (A) or in co-culture with U266B1 (B). Numbers of MN in HS5 cells following exposure to thalidomide or lenalidomide increased at each time point compared to the control when cultured independently. Numbers of MN following exposure to bortezomib remained consistent to that of the control. Carfilzomib exposure gave rise to an increase in MN at each time point but was not significantly different to the control. Co-culture with U266B1 cells caused a small decrease in the number of MN in thalidomide exposed cultures compared to when cultured alone. Lenalidomide exposed cells had remained at levels consistent with that of independent culture. Treatment with bortezomib or carfilzomib did not significantly alter the levels of MN compared to the control in co-culture with HS5. Samples taken at 1, 24, 48 and 72 hrs post exposure. Bars represent mean ± SD (n=3).

MN in U266B1 cells fluctuated after exposure to each of the chemotherapeutic agents at each time point (figure 5.20). RICC calculated for U266B1 cells cultured alone and in co-culture with HS5 cells can be seen in table 5.4. MN increased up to 48 hrs following exposure to thalidomide but were not significantly increased compared to the control at any time point. Lenalidomide and bortezomib exposure did not significantly increase MN at any time point. Carfilzomib treatment increased MN at each time point when U266B1 were cultured independently but was not significantly higher compared to the control ($p>0.05$).

Numbers of MN in U266B1 cells were lower when co-cultured with HS5 cells. U266B1 cells exposed to thalidomide in co-culture with HS5 had an initial increase in MN before decreasing at subsequent time points. Lenalidomide did not significantly increase numbers of MN in U266B1 cells at any time point when administered to the co-culture. There was a rise in MN at 48 and 72 hrs post exposure to bortezomib but this was not significant when compared to the control ($p>0.05$). Carfilzomib also increased numbers of MN at each time point but was not considered significant compared to the control ($p>0.05$).

Time (Hours)	Relative Increase in Cell Counts (RICC) (%)							
	Thalidomide	Lenalidomide	Bortezomib	Carfilzomib	Thalidomide	Lenalidomide	Bortezomib	Carfilzomib
	Alone	Alone	Alone	Alone	Co-culture	Co-culture	Co-culture	Co-culture
1	97	98	58	66	80	50	55	57
24	100	98	46	88	67	50	9.2	33
48	100	100	50	42	100	100	2.9	27
72	93	100	30	30	100	100	3.5	43

Table 5.4 RICC of U266B1 cells cultured independently or in co-culture with HS5 cells. Thalidomide and lenalidomide did not cause the RICC of U266B1 cells to be below 50% at any time point when cultured independently or when in co-culture with HS5. RICC were less than 50% at 72 hrs following brotzomib exposure when cultured alone. Carfilzomib caused RICC to be below 50% at 48 and 72 hrs when cultured independently. Both proteasome inhibitors gave rise to an RICC below 50% at 24, 48 and 72 hrs when co-cultured with HS5 cells.

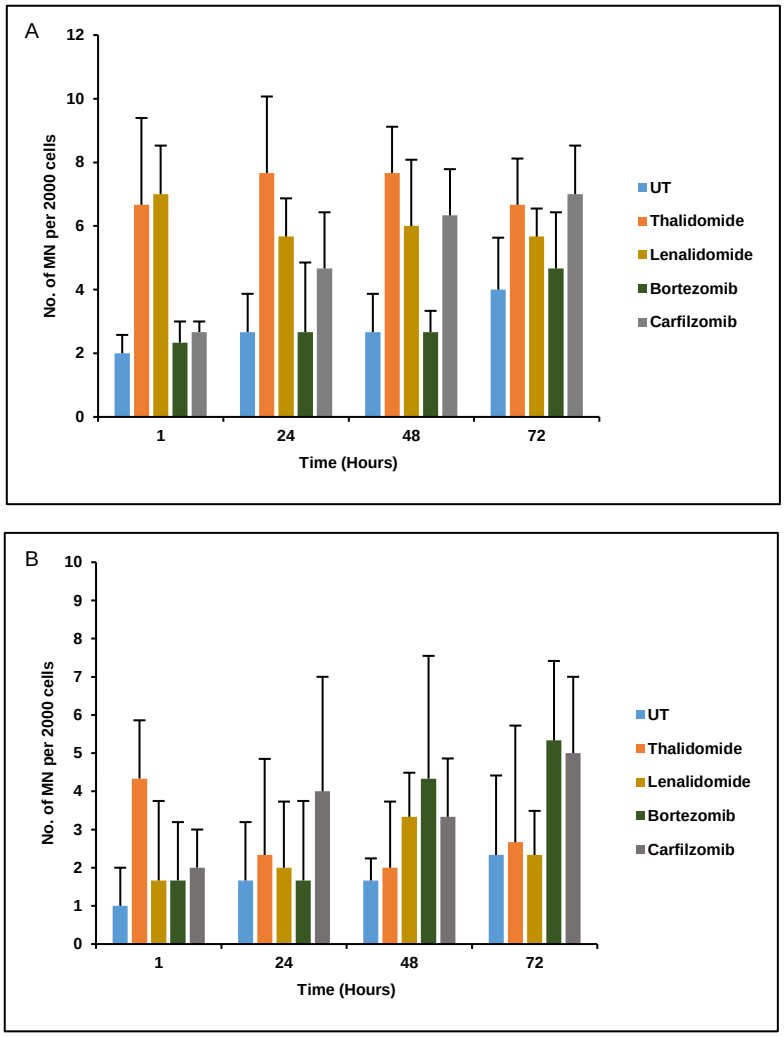

Figure 5.20 Numbers of micronuclei (MN) in U266B1 cells over a 72 hr period following exposure to chemotherapy. U266B1 cells were exposed to either thalidomide (200 ng / ml), lenalidomide (4 µM), bortezomib (500 nM), carfilzomib (13.8 nM) or left untreated. U266B1 cells were exposed to each chemotherapeutic agent for 1 hr either alone (A) or in co-culture with HS5 (B). Numbers of MN in U266B1 cells increased following exposure to thalidomide when cultured independently up to 48 hrs. Lenalidomide or bortezomib treatment did not significantly increase numbers of MN in independent cultures. Carfilzomib administered to alone and co-cultures gave rise to an increase in MN at each time point. U266B1 cells exposed to thalidomide in co-culture with HS5 had an initial increase in MN before decreasing at subsequent time points. Bortezomib increased numbers of MN at 48 and 72 hrs in co-cultures. Samples taken at 1, 24, 48 and 72 hrs post exposure. Bars represent mean ± SD (n=3).

HS5 and U266B1 cells were cultured independently and co-cultured together via an insert and exposed to either thalidomide (200 ng / ml), lenalidomide (4 µM), bortezomib (500 nM), carfilzomib (13.8 nM) or left untreated. Cells were measured for BN at 1, 24, 48 and 72 hrs post exposure. Typical images of BN U266B1 and HS5 cells can be seen in figure 5.21. RICC calculated for HS5 cells cultured alone or in co-culture with U266B1 cells can be seen in table 5.3.

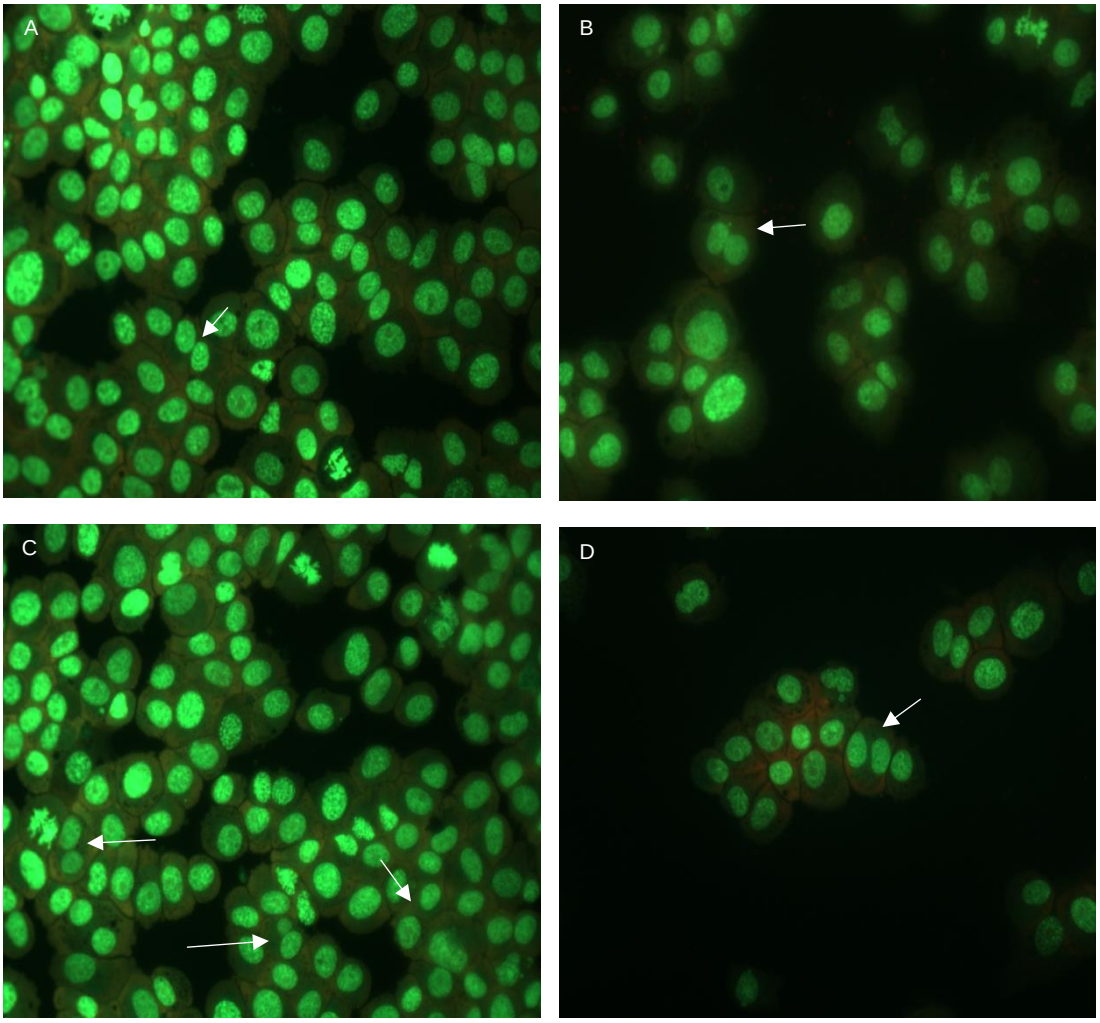

Figure 5.21 Representative images of bi-nucleated HS5 and U266B1 cells. HS5 and U266B1 cells were cultured independently and exposed to either bortezomib (500 nM), carfilzomib (13.8 nM) for 1 hr or left untreated. Cells were harvested and stained with acridine orange. Numbers of bi-nucleated cells per 2000 mono-nuclear cells were scored. A) HS5 cells cultured independently and left untreated at 72 hr, B) U266B1 cells cultured independently and exposed to carfilzomib, 24 hrs post exposure. C) HS5 cells cultured independently and exposed to carfilzomib, 24 hrs post exposure. D) U266B1 cells cultured independently and exposed to bortezomib, 72 hrs post exposure Arrows indicate bi-nucleated cells (x 40 magnification).

Numbers of BN HS5 cells increased following exposure to chemotherapy when cultured alone and in co-culture with U266B1 cells (figure 5.22). BN in HS5 cells exposed to each of the agents increased at 24, 48 and 72 hrs when cells were cultured independently. BN were significantly higher compared to the control after exposure to each of the agents at 48 hrs ($p<0.05$). Lenalidomide, bortezomib and carfilzomib at 72 hrs had significantly high numbers of BN compared to the control ($p<0.05$) when cultured alone.

Numbers of BN also increased compared to the control when HS5 cells were co-cultured with U266B1 cells and exposed to chemotherapy. BN were significantly higher compared to the control after exposure to lenaldiomide in co-culture at 48 hrs ($p<0.05$). Numbers of BN following exposure to each of the agents were not significantly higher than the control at any time point. Lenalidomide treatment gave rise to the highest numbers of BN in HS5 cells that were co-cultured.

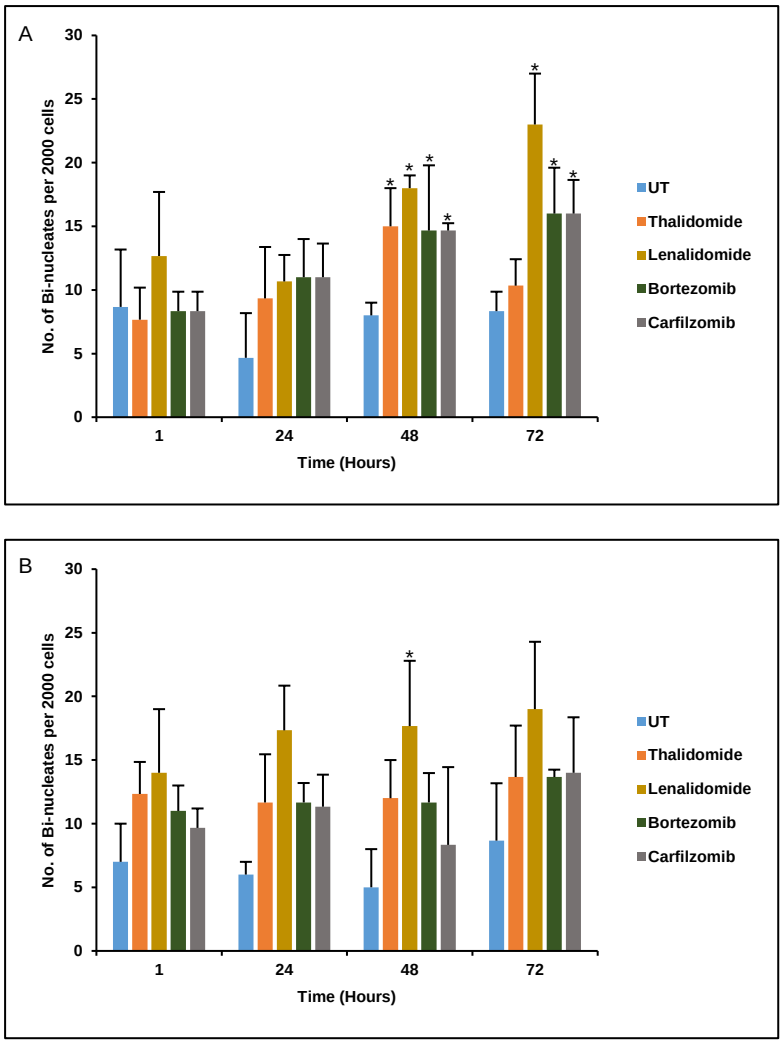

Figure 5.22 Numbers of bi-nucleated (BN) cells over a 72 hr period in HS5 stromal cells following exposure to chemotherapy. HS5 cells were exposed to either thalidomide (200 ng / ml), lenalidomide (4 µM), bortezomib (500 nM), carfilzomib (13.8 nM) or left untreated. HS5 stromal cells were exposed to each chemotherapeutic agent for 1 hr either alone (A) or in co-culture with U266B1 (B). Numbers of BN cells were higher when cultured independently compared to when co-cultured with U266B1 cells following exposure to chemotherapy. Numbers of BN cells were highest in independent and co-cultures exposed to lenalidomide. Samples taken at 1, 24, 48 and 72 hrs post exposure. Bars represent mean ± SD (n=3) (*p<0.05).

RICC calculated for U266B1 cells cultured alone and in co-culture with HS5 cells can be seen in table 5.4. Numbers of BN U266B1 cells increased when exposed to chemotherapy and cultured independently compared to the control (figure 5.23). Thalidomide had significantly higher numbers of BN compared to the control 72 hrs post exposure ($p<0.05$). Lenalidomide had significantly higher BN at 24 ($p<0.01$), 48 ($p<0.05$) and 72 hrs post exposure compared to the control ($p<0.01$). Bortezomib gave rise to increased BN at 72 hrs ($p<0.01$). Carfilzomib did not significantly increase BN in HS5 cells when compared to the control ($p<0.05$).

When in co-culture with HS5 cells, numbers of BN continued to increase at each time point when exposed to lenalidomide (figure 5.22) with BN significantly higher at 24 and 72 hrs ($p<0.01$) compared to the untreated control. U266B1 cells exposed to each of the agents in co-culture had significantly higher BN at 24 hrs post exposure ($p<0.05$). Numbers of BN after exposure to bortezomib increased at each time point but were not significantly higher compared to the control at 48 and 72 hrs. Numbers of BN cells increased at 24 hrs post exposure to carfilzomib but decreased to levels similar to that of the control at 48 and 72 hrs.

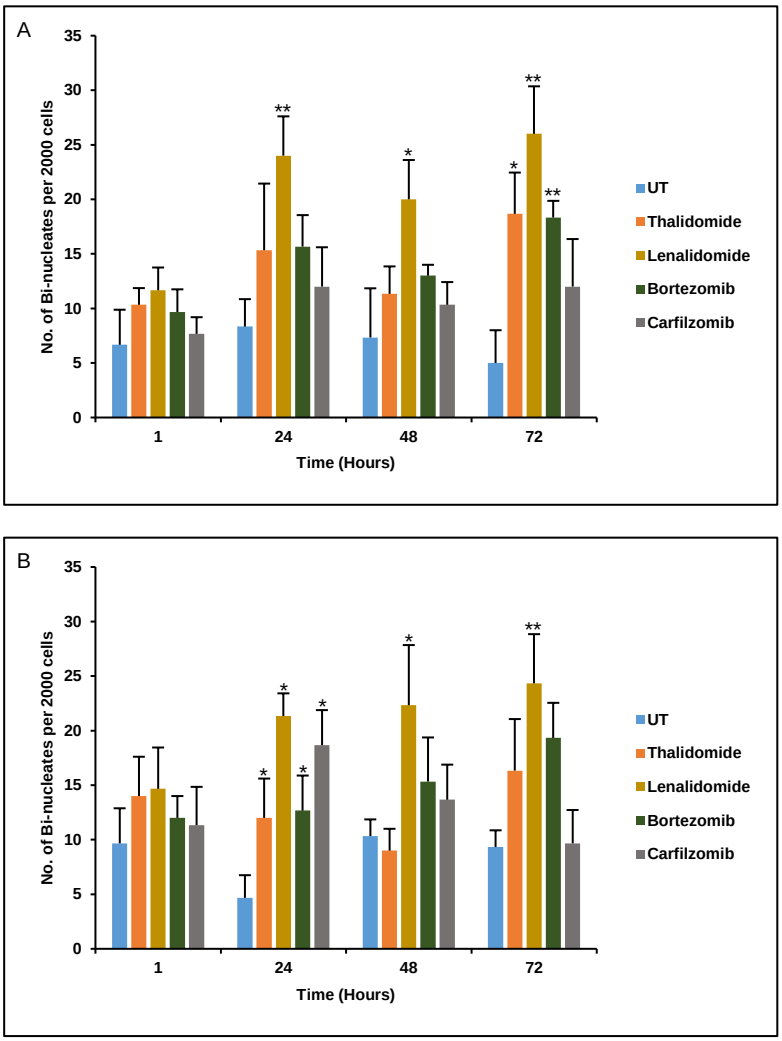

Figure 5.23 Numbers of bi-nucleated (BN) cells over a 72 hr period in U266B1 cells following exposure to chemotherapy. U266B1 cells were exposed to either thalidomide (200 ng / ml), lenalidomide (4 µM), bortezomib (500 nM), carfilzomib (13.8 nM) or left untreated. U266B1 cells were exposed to each chemotherapeutic agent for 1 hr either alone (A) or in co-culture with U266B1 (B). Levels of BN cells increased when exposed to chemotherapy and cultured independently compared to the control. When in co-culture with HS5 cells, numbers of BN remained at levels reported when cultured independently. Lenalidomide exposure resulted in the highest number of BN at each time point when administered to alone and co-cultures. Samples taken at 1, 24, 48 and 72 hrs post exposure. Bars represent mean ± SD (n=3) (*p<0.05, **p<0.01).

HS5 and U266B1 cells were cultured independently and co-cultured together via an insert and exposed to either thalidomide (200 ng / ml), lenalidomide (4 µM), bortezomib (500 nM), carfilzomib (13.8 nM) or left untreated. Cells were measured for multi-nucleation at 1, 24, 48 and 72 hrs post exposure. RICC calculated for HS5 cells cultured alone or in co-culture with U266B1 cells can be seen in table 5.3.

Following exposure to chemotherapy it was noted that a number of HS5 cells contained three or more nuclei (figure 5.24).

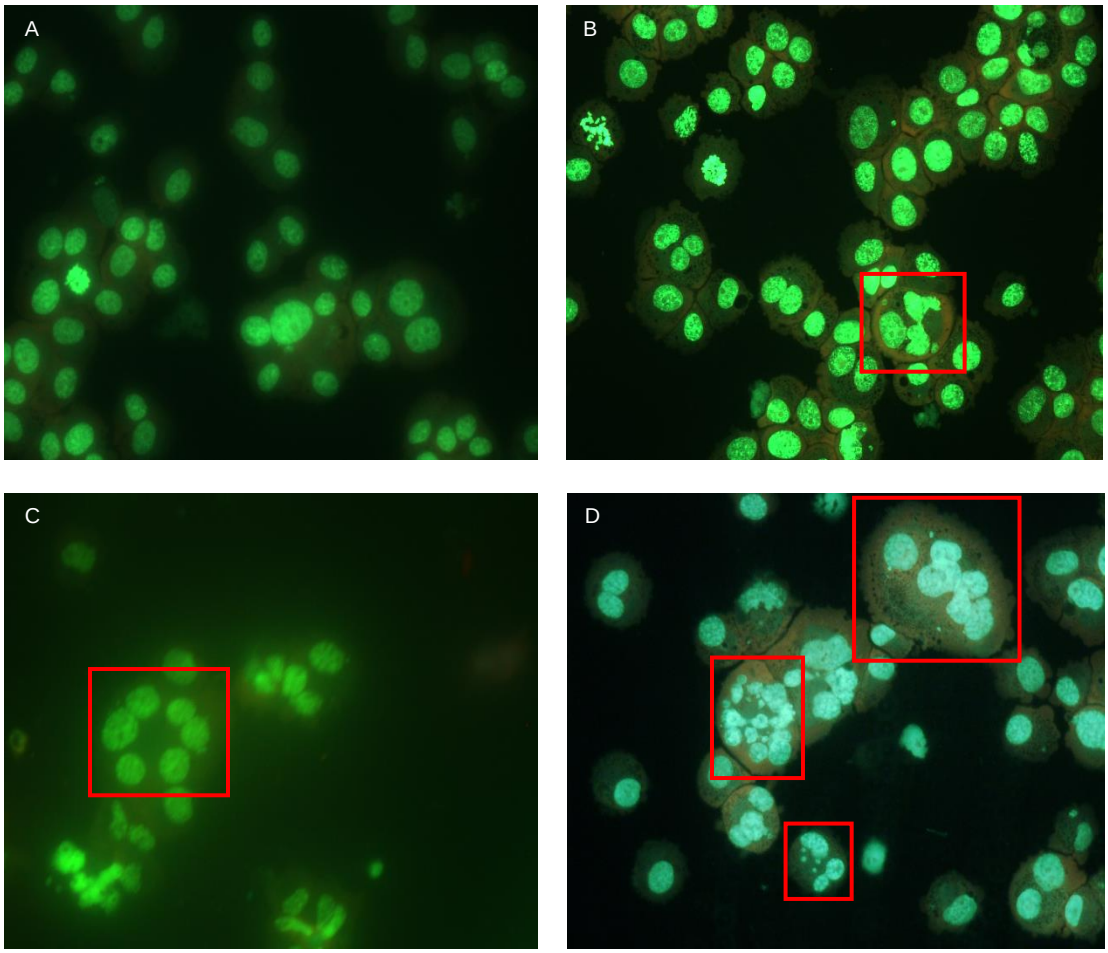

Figure 5.24 Representative images of multi-nucleated HS5 cells. A) HS5 cells cultured independently and left untreated at 24 hrs, B) HS5 cells co-cultured with U266B1 and exposed to carfilzomib (13.8 nM) image taken at 48 hrs post exposure, C) HS5 cells independently cultured and exposed to lenalidomide (4 µM) at 1 hr post exposure, D) HS5 cells co-cultured with U266B1 48 hrs post exposure to thalidomide (200 ng / ml). Red boxes indicate multi-nucleated cells (x 40 magnification).

These multi-nucleated HS5 cells increased in number in a time dependent manner following exposure to thalidomide when cells were cultured alone (figure 5.25). Multi-nucleated cells in the untreated control averaged between 2 and 4 per 2000 cells at each time point. Multi-nucleated HS5 cells were significantly higher than that of the control at 24 ($p<0.01$), 48 ($p<0.01$) and 72 hrs ($p<0.01$) post exposure to thalidomide. Multi-nucleation was also seen in cells exposed to lenalidomide and bortezomib, with multi-nucleation also increasing at each time point post-exposure. Multi-nucleation in HS5 cells exposed to lenalidomide were also significantly higher at 24 ($p<0.001$), 48 ($p<0.01$) and 72 hrs ($p<0.001$) post-exposure compared to the control. Multi-nucleated cells were significantly higher at 24 ($p<0.05$), 48 ($p<0.05$) and 72 hrs ($p<0.05$) following exposure to bortezomib. Carfilzomib exposure did not significantly increase the multi-nucleation in HS5 cells at any time point ($p>0.05$).

HS5 cells that were multi-nucleated were reduced when in co-culture with U266B1. Thalidomide exposed co-cultures had significantly higher multi-nucleation at 1 ($p<0.05$), 24 ($p<0.01$) and 48 hrs ($p<0.05$) compared to untreated. Lenalidomide exposed co-cultures increased multi-nucleation in HS5 cells significantly at 24 hrs ($p<0.05$) post-exposure. Bortezomib significantly increased multi-nucleation in HS5 cells at 24 hrs post treatment ($p<0.05$) but were not significantly higher at subsequent time points. Carfilzomib exposure did not significantly increase multi-nucleation in HS5 cells in co-culture with U266B1 ($p<0.05$).

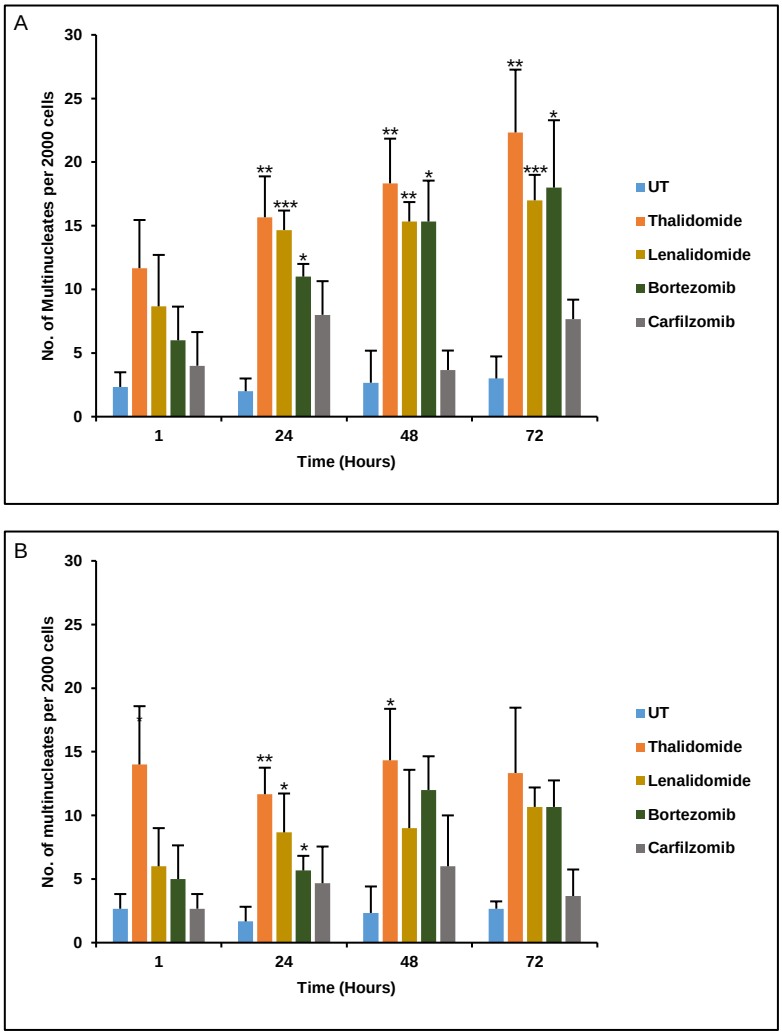

Figure 5.25 Numbers of multi-nucleated HS5 cells following exposure to chemotherapy over a 72 hr period. HS5 cells were exposed to either thalidomide (200 ng / ml), lenalidomide (4 µM), bortezomib (500 nM), carfilzomib (13.8 nM) or left untreated. HS5 stromal cells were exposed to each chemotherapeutic agent for 1 hr either alone (A) or in co-culture with U266B1 (B). Numbers of multi-nucleated cells (cells with 3 or more nuclei) increased at each time point following exposure to thalidomide, lenalidomide or bortezomib when cultured independently. Numbers of multi-nucleated cells were reduced following exposure to these agents when co-cultured with U266B1 cells. Samples taken at 1, 24, 48 and 72 hrs post exposure. Bars represent mean ± SD (n=3) (*p<0.05, **p<0.01, ***p<0.001).

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RICC calculated for U266B1 cells cultured alone and in co-culture with HS5 cells can be seen in table 5.4. U266B1 cells exposed to each of the chemotherapeutic agents also gave rise to increased numbers of multi-nucleated cells in a time dependent manner (figure 5.26). Representative images of these abnormal multi-nucleated U266B1 cells can be seen in figure 5.27. Each of the chemotherapeutic agents increased numbers of multi-nucleated cells at 1 hr post exposure ($p<0.05$) in independent cultures. Multi-nucleated cells in the untreated control averaged between 1 and 4 per 2000 cells at each time point. Levels of multi-nucleated cells were significantly higher than the control at 1 hr following treatment with all agents ($p<0.05$) and 24 hrs post exposure to all agents except carfilzomib ($p<0.05$). Multi-nucleation was significantly higher than the control at 48 hrs following thalidomide ($p<0.01$), lenalidomide ($p<0.01$), bortezomib ($p<0.01$) and carfilzomib ($p<0.001$) treatment. Multi-nucleation continued to increase at 72 hrs post exposure to each of these agents and was significantly higher than the control following thalidomide ($p<0.01$), lenalidomide ($p<0.01$), bortezomib ($p<0.05$) and carfilzomib ($p<0.05$) treatment.

Numbers of multi-nucleated U266B1 cells were lower when in co-culture with HS5 (figure 5.26, B). Thalidomide treatment increased multi-nucleation at 1hr ($p<0.01$) which then decreased at 24 and 48 hrs although still remained higher than the control. Numbers of multi-nucleated cells then increased again at 72 hrs ($p<0.01$). Lenalidomide exposure increased multi-nucleation at each time point and was significantly higher than the control at 72 hrs ($p<0.01$). Bortezomib treatment increased multi-nucleation at 72 hrs ($p<0.05$). Carfilzomib exposure did not significantly increase the numbers of multi-nucleated cells at any time point.

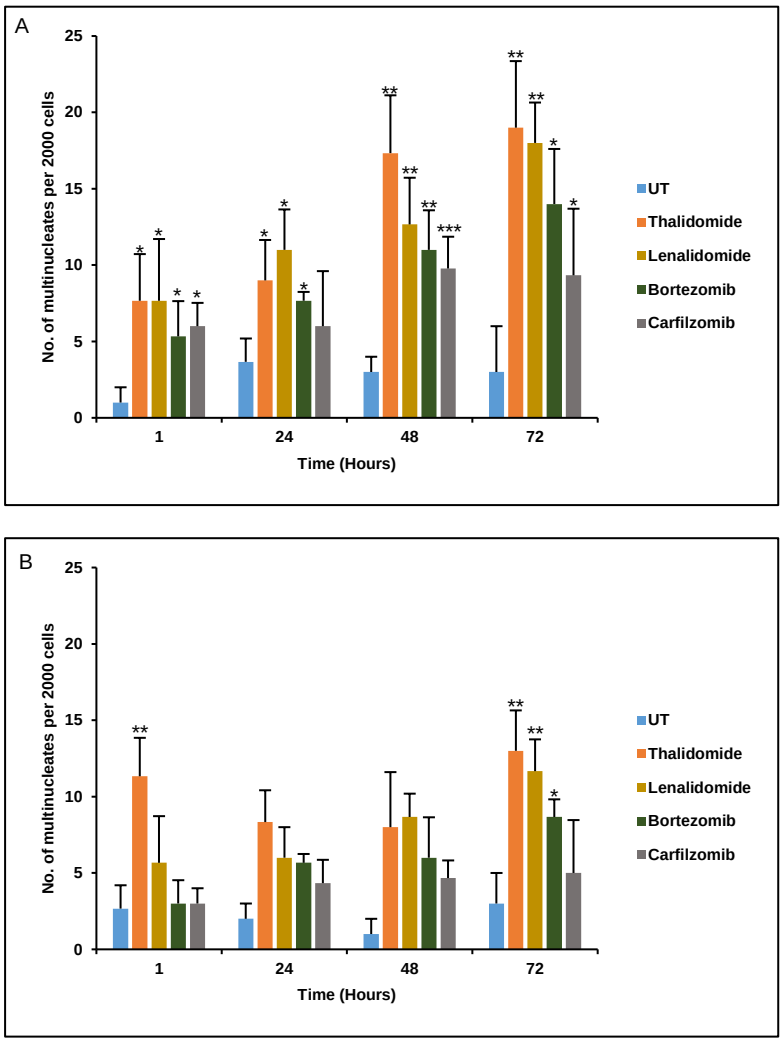

Figure 5.26 Numbers of multi-nucleated cells over a 72 hr period in U266B1 cells following exposure to chemotherapy. U266B1 cells were exposed to either thalidomide (200 ng / ml), lenalidomide (4 µM), bortezomib (500 nM), carfilzomib (13.8 nM) or left untreated. U266B1 cells were exposed to each chemotherapeutic agent for 1 hr either alone (A) or in co-culture with U266B1 (B). Numbers of multi-nucleated cells increased at each time point following exposure to chemotherapeutic agents when cultured independently. Numbers of multi-nucleated cells were lower when exposed to chemotherapy in a co-culture with HS5 cells. Samples taken at 1, 24, 48 and 72 hrs post exposure. Bars represent mean ± SD (n=3) (*p<0.05, **p<0.01).

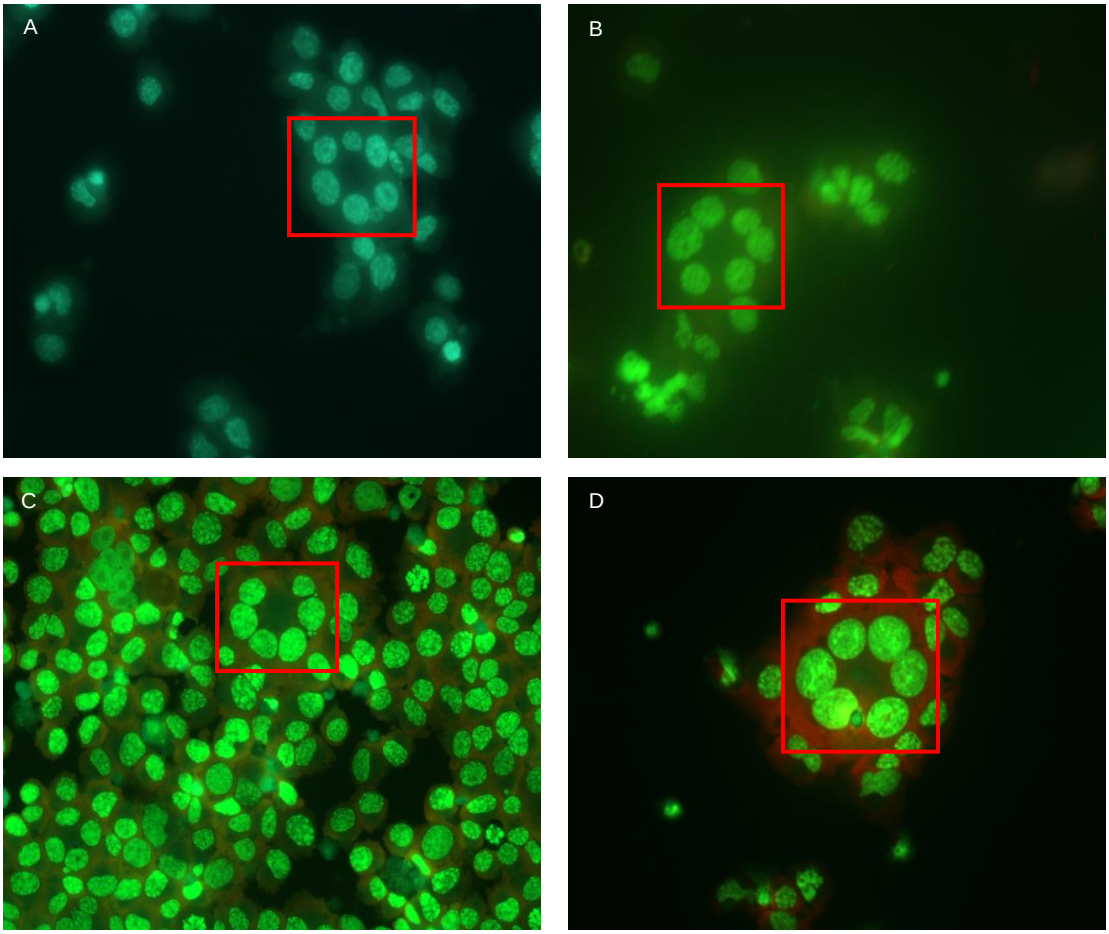

Figure 5.27 Representative images of multi-nucleated U266B1 cells following exposure to chemotherapy. All cells were treated with chemotherapy for 1 hr and stained using acridine orange. A) U266B1 cells cultured independently and exposed to thalidomide (200 ng / ml) 24 hrs post exposure, B) U266B1 cells co-cultured with HS5 and exposed to carfilzomib (13.8 nM) 24 hrs post exposure, C) U266B1 cells independently cultured and exposed to lenalidomide (4 µM) at 24 hr post exposure, D) U266B1 cultured independently and exposed to bortezomib (500 nM) 72 hrs post exposure. Red boxes indicate multi-nucleated cells (x 40 magnification).

To investigate whether a genotoxic effect could be passed from one cell to another (bystander effect) HS5 and U266B1 cells respectively were exposed to chemotherapy for 1 hr and then co-cultured with previously unexposed U266B1 or HS5 cells for 72 hrs. Following the increase in multi-nucleation observed after treatment with some of the agents, each cell line was measured for multi-nucleation at 1, 24, 48 and 72 hrs following a co-culture. RICC calculated for HS5 and U266B1 cells can be seen in table 5.5 and 5.6 respectively.

Multi-nucleation in HS5 cells that were exposed to thalidomide and co-cultured with untreated U266B1 were not significantly different to HS5 cells that were not exposed to drug and co-cultured with thalidomide exposed U266B1 (figure 5.28). Numbers of multi-nucleated cells were highest in cells exposed directly to thalidomide compared to any other agent. Multi-nucleation increased at each time point in HS5 cells exposed directly to lenalidomide and co-cultured with untreated U266B1 cells. However they were not significantly different to HS5 cells that were not exposed to lenalidomide. These findings suggest that a bystander effect does occur between these two cell types when MM cells are exposed to immunomodulatory agents

HS5 cells not exposed to bortezomib directly and co-cultured with previously treated U266B1 did not produce significant multi-nucleation. HS5 cells treated with bortezomib and co-cultured with untreated U266B1 cells had increased multi-nucleation at each time point and were significantly higher than HS5 cells not exposed to bortezomib at 48 hrs ($p<0.01$). HS5 cells exposed to carfilzomib directly or via U266B1 cells did not give rise to a significant increase in multi-nucleation at any time point. Numbers of multi-nucleated cells were similar to that observed previously (figure 5.25, B) when both HS5 and U266B1 cells were exposed to drug directly in co-culture.

Time (Hours)	Relative Increase in Cell Counts (RICC) (%)			
	Thalidomide	Lenalidomide	Bortezomib	Carfilzomib
1	100	90	92	100
24	63	100	68	67
48	58	83	45	70
72	45	79	30	45

Table 5.5 RICC of HS5 cells in bystander model. RICC was below 50% at 72 hrs following exposure to bortezomib. All other agents gave rise to an RICC above 50% at each time point.

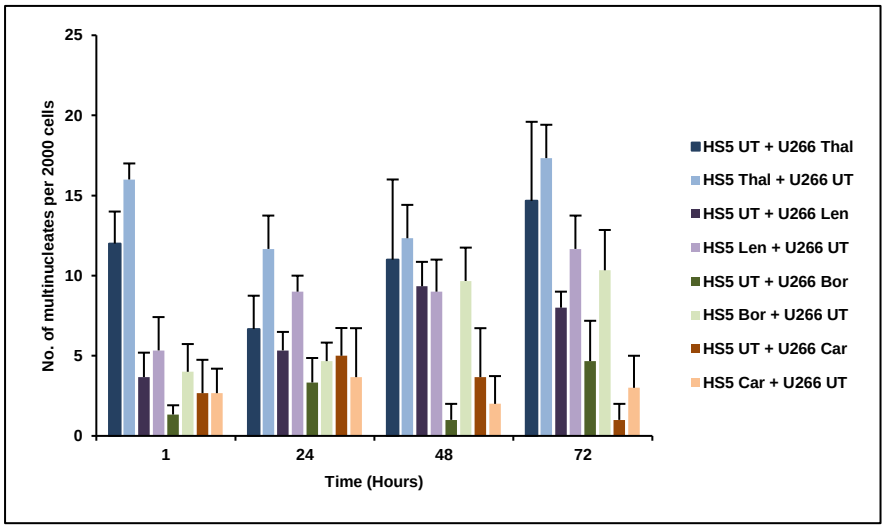

Figure 5.28 Numbers of multi-nucleated HS5 cells following exposure to chemotherapy either directly or when in culture with previously exposed U266B1 cells over a 72 hr period. HS5 cells were exposed to either thalidomide (200 ng / ml), lenalidomide (4 µM), bortezomib (500 nM), carfilzomib (13.8 nM) or left untreated. HS5 stromal cells were exposed to each chemotherapeutic agent for 1 hr before co-culturing with untreated U266B1 cells. HS5 cells that were not exposed to drug were co-cultured with previously treated U266B1 cells. Untreated HS5 co-cultured with previously thalidomide exposed U266B1 cells had numbers of multi-nucleated cells consistent with cells that were directly exposed to drug. Numbers of multi-nucleated cells in lenalidomide exposed HS5 directly and indirectly were also not significantly different with a rise in multi-nucleation at 48 hrs. Bortezomib exposed HS5 cells had increased multi-nucleation at each time point. Cells not directly exposed to bortezomib did not have a significant rise in multi-nucleation. Carfilzomib did not produce significant multi-nucleation. Samples taken at 1, 24, 48 and 72 hrs post exposure. Bars represent mean ± SD (n=3).

U266B1 cells that were untreated and co-cultured with HS5 cells previously exposed to thalidomide had an increase in multi-nucleation at each time point (figure 5.29). At 48 and 72 hrs previously untreated U266B1 had higher numbers of multi-nucleated cells compared to U266B1 cells that were originally exposed to thalidomide. Each of the other U266B1 cells exposed to chemotherapy had increased multi-nucleation compared to the untreated cells. Untreated U266B1 cells had numbers of multi-nucleated cells consistent with that of untreated Controls when U266B1 cells were cultured alone for 72 hrs (figure 5.26, A).

Time (Hours)	Relative Increase in Cell Counts (RICC) (%)			
	Thalidomide	Lenalidomide	Bortezomib	Carfilzomib
1	60	80	47	50
24	67	90	41	67
48	100	64	35	86
72	100	75	50	75

Table 5.6 RICC of U266B1 cells in bystander model. RICC was below 50% at 24 and 48 hrs following exposure to bortezomib. All other agents gave rise to an RICC above 50% at each time point.

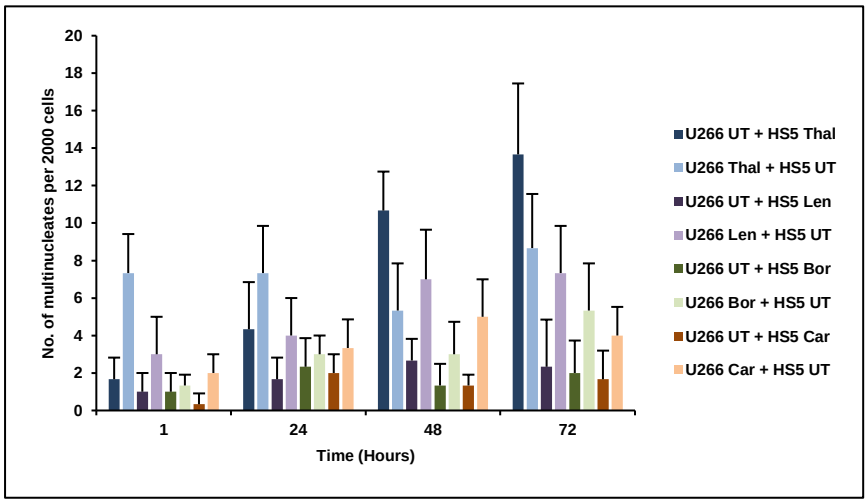

Figure 5.29 Numbers of multi-nucleated U266B1 cells following exposure to chemotherapy either directly or when in culture with previously exposed HS5 cells over a 72 hr period. U266B1 cells were exposed to either thalidomide (200 ng / ml), lenalidomide (4 µM), bortezomib (500 nM), carfilzomib (13.8 nM) or left untreated. U266B1 cells were exposed to each chemotherapeutic agent for 1 hr before co-culturing with untreated HS5 cells. U266B1 cells that were not exposed to drug were co-cultured with previously chemotherapy treated HS5 cells. Untreated U266B1 cells that were co-cultured with previously thalidomide treated HS5 had an increase in multi-nucleation at each time point. Highest multi-nucleation was seen in these untreated cells indicating a bystander effect between the two cell types. Multi-nucleation was highest in all other U266B1 cells that were exposed to chemotherapy directly. All other untreated U266B1 cells cultured with chemotherapy treated HS5 cells did not have a significant rise in multi-nucleation. Samples taken at 1, 24, 48 and 72 hrs post exposure. Bars represent mean ± SD (n=3).

5.3.3 Synchronisation of TK6 lymphoblast cells

Following the observations of increased multi-nucleation in HS5 and U266B1 cells, Ms Jennifer Razik, an undergraduate student at UWE also reported an increase of multi-nucleated cells in TK6 lymphoblast cells following thalidomide and lenalidomide exposure. As such, attempts were made to synchronise TK6 cells and expose them to the clinical doses of these agents used in the MM model. All work in the section was performed in conjunction with Ms Jennifer Razik and supervised by me.

Flow cytometry was employed (figure 5.30) to analyse phases of the cell cycle, and to determine at which time point a high percentage of cells were observed in G2-M phase. Table 5.7 shows average percentage of cells found in each phase, and shows that at 9 hrs after final wash/release of thymidine, a high percentage of cells were travelling in mitosis.

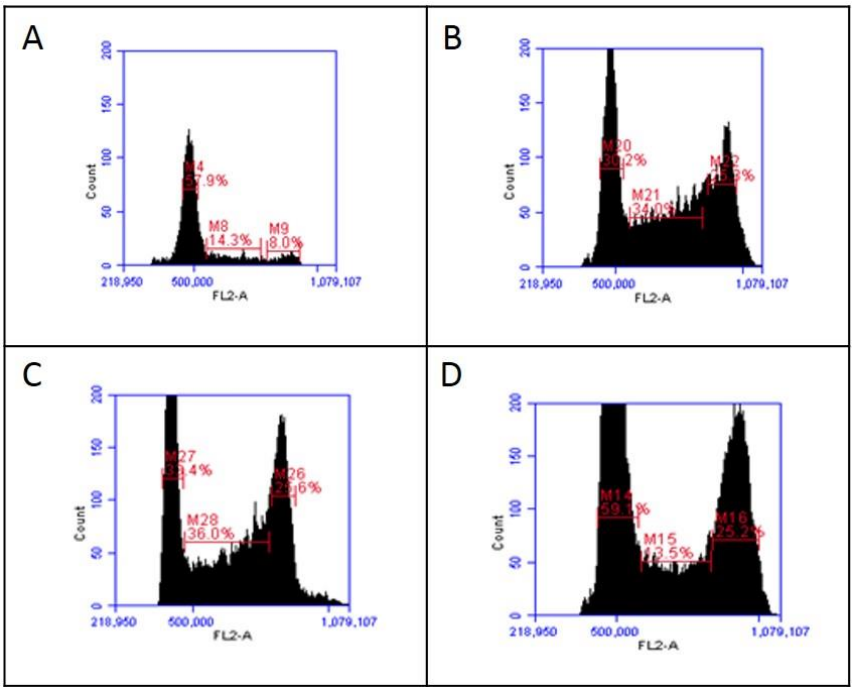

Figure 5.30 Flow cytometry analysis of cell cycle in TK6 cells. Following a double thymidine-block, cells were stained using propidium iodide and cell cycle analysis using S-phase assessment was completed. Images were taken using BD Accuri C6™, CFlow plus to show different phases of cell cycle. Time after second-thymidine block shows A) 1 hour, cells are mostly at G0/G1 B) 4 hrs is S-phase C) 6 hrs is G2 D) 9 hrs is G2-M-phase.

Table 5.7 Percentage of TK6 cells in each phase of cell cycle.

Time point (hrs) / Phase	G1	GS	G2/M
4	37.8%	19.25%	23.7%
6	39.9%	33.8%	24.1%
9	47.8%	18.9%	31.8%

Table 5.7 shows percentage of cells in each phase of cell cycle, following a second-thymidine block release, at 4, 6 and 9 hrs. Data was obtained using BD Accuri C6™ CFlow plus (n=4).

5.3.4 Cell synchronisation impact on induction of multi-nucleation following exposure to thalidomide and lenalidomide

Following synchronisation of TK6 cells with a double thymidine block, cells were recorded for BN (figure 5.31) and multi-nucleated (figure 5.32) cells after treatment with thalidomide (200 ng / ml) and lenalidomide (4 µM). Cells were scored at 3, 5, 24 and 48 hrs post exposure to drug and compared to untreated cells. TK6 have a cell cycle of 16 - 20 hrs (Liviac *et al*., 2010), therefore, effects seen over 24 hrs confirm cells have undergone at least 1 complete cell division.

Thalidomide treatment resulted in a significant increase in BN cells at each time point (figure 5.31). Results observed found that BN and multi-nucleation induced by thalidomide and lenalidomide exposure significantly increased in a time dependent manner. BN of TK6 cells was significant at 3 hrs ($p<0.01$), 5 hrs ($p<0.001$), 24 ($p<0.01$) and 48 hrs ($p<0.001$) post exposure compared to the untreated. Lenalidomide treatment also resulted in a significant increase in BN cells at 5 ($p<0.001$), 24 ($p<0.01$) and 48 hrs ($p<0.01$) post exposure compared to the untreated.

Time (Hours)	Relative Increase in Cell Counts (RICC) (%)	
	Thalidomide	Lenalidomide
3	57	55
5	60	67
24	81	67
48	80	75

Table 5.8 RICC of TK6 cells treated with chemotherapy following cell synchronisation. Both thalidomide and lenalidomide recorded an RICC above 50% at each time point.

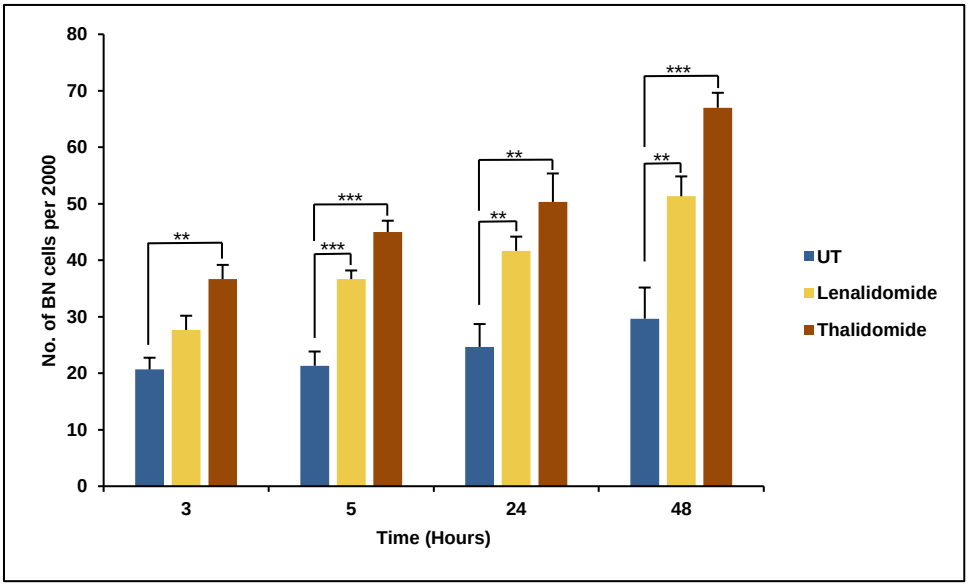

Figure 5.31 Frequency of bi-nucleation on TK6 cells following cell synchronisation and exposure to immunomodulatory agents. Treatment of synchronised TK6 cells with immunomodulatory agents resulted in an increase in bi-nucleated cells at each time point. Samples taken at 3, 5, 24, 48 hrs post exposure to either lenalidomide (4 µM) or thalidomide (200 ng / ml) (n=3). Bi-nucleates were scored per 2000 mononuclear cells. Bars represent mean ± SD n=3 (** $p<0.01$, *** $p<0.001$).

Thalidomide treatment also resulted in a significant increase in multi-nucleated cells at each time point (figure 5.32). At 3 hrs ($p<0.001$), 5 hrs ($p<0.01$), 24 ($p<0.01$) and 48 hrs ($p<0.001$) post exposure compared to the untreated. Lenalidomide treatment similarly resulted in a significant increase in multi-nucleated cells at 5 ($p<0.001$), 24 ($p<0.01$) and 48 hrs ($p<0.001$) post exposure compared to the untreated.

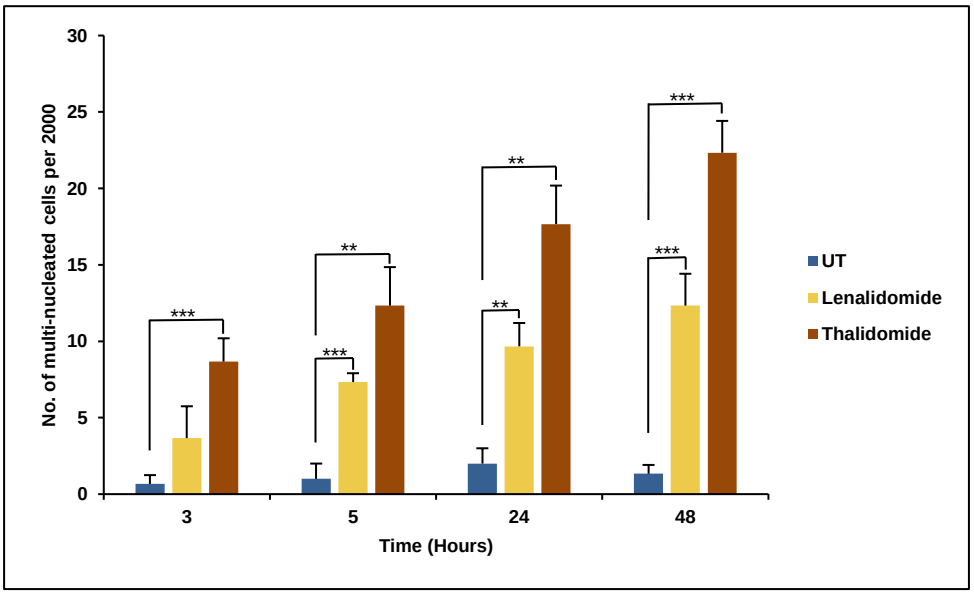

Figure 5.32 Frequency of multi-nucleation on TK6 cells following cell synchronisation and exposure to immunomodulatory agents. Treatment of synchronised TK6 cells with immunomodulatory agents resulted in an increase in multi-nucleated cells at each time point. Samples taken at 3, 5, 24, 48 hrs post exposure to either lenalidomide (4 µM) or thalidomide (200 ng / ml) (n=3). Multi-nucleated cells were scored per 2000 mononuclear cells. Bars represent mean ± SD n=3 (** $p<0.01$, *** $p<0.001$).

Following the cell synchronisation protocol and relevant treatments, several cells with aberrant nuclei were observed (figure 5.33), for both agents.

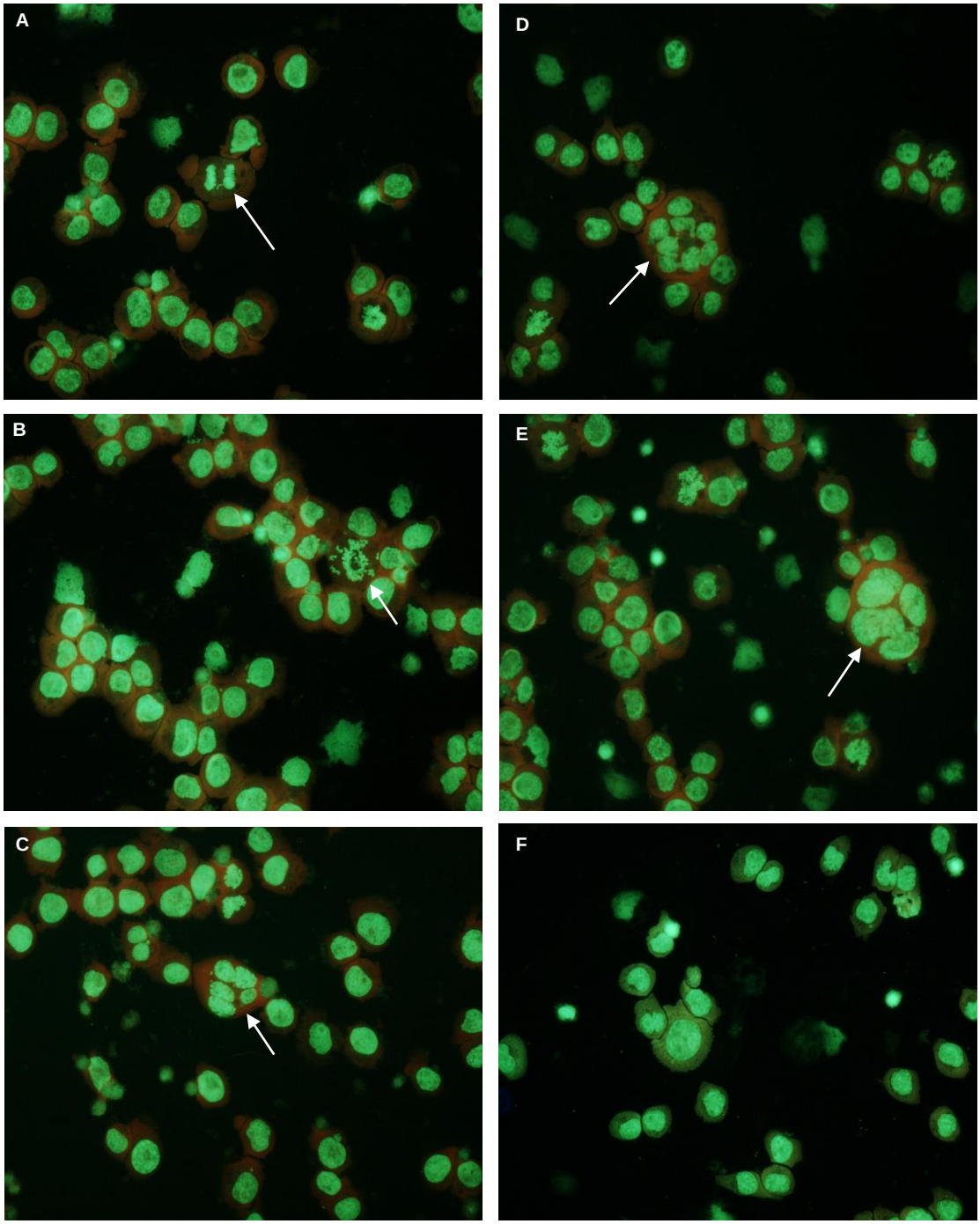

Figure 5.33 Representative images of bi-nucleated and multi-nucleated on synchronised TK6 cells following thalidomide or lenalidomide exposure. All cells were treated with either thalidomide (200 ng / ml) (A, B, C) or lenalidomide (4 µM) (D, E) for 1 hr following a thymidine double block. A) Bi-nucleated cell with anaphase bridging, 3 hrs post treatment, B) Aberrant mitosis, 5 hrs post treatment, C) Multi-nucleated cell with 5 nuclei, 24 hrs post treatment, D) Multi-nucleated cell with 8 nuclei, 48 hrs post treatment, E) Multi-nucleated cell with 5 nuclei, 72 hrs post treatment, F) Represents a typical control image at 3 hrs (40 x magnification).

5.4 Discussion

As discussed previously, the chemotherapeutic agents used in the treatment of MM have wide ranging mechanisms of action. Some agents such as melphalan are known to directly target DNA (Huang and Li, 2013) while others such as the immunomodulatory agents have been documented to affect DNA indirectly via the production of ROS (Aerbajinai *et al*., 2007) or through the suppression of DNA repair mechanisms by proteasome inhibitors (Murakawa *et al*., 2007). The genotoxic effects of chemotherapeutic agents in an *in vitro* co-culture model of disease are less well reported. Furthermore, while it is known that MM cells are protected from chemotherapy by BM-MSC, whether this protection occurs at a DNA level and what the implications may be for the MSC is not known. Detection of DNA damage is a useful tool to investigate the efficacy and toxicity of drugs as well as monitoring DNA damage vs DNA repair over a specific time frame after treatment (Olive and Banáth, 2006).

In this chapter a series of experiments were carried out with the aim of elucidating the potential genotoxic effects of chemotherapeutic agents used in MM on both BM-MSC/HS5 and U266B1 cells when in a non-contact co-culture model (chapter 3). Clinically, DNA damage has the potential to invoke a number of serious ramifications, particularly in MSC which could contribute to a loss of function or an increased risk of future malignancies. In this study both *in vitro* comet and micronucleus assays were used to detect for genotoxicity within the model. Each of these methods measure different endpoints of DNA damage although both tests generate complementary information and may be used for the comparison of identical treatment conditions in cells *in vitro* and *in vivo*. Furthermore this chapter also reports on the formation of multinucleated cells as a result of exposure to immunomodulatory agents thalidomide and lenalidomide.

5.4.1 Assessment of genotoxic damage following chemotherapeutic treatment using the comet assay

The comet assay is a rapid and sensitive method for the detection of DNA damage in a range of mammalian cell types (Ribas-Maynou *et al*., 2012; Kopjar *et al*., 2006) as well as plants (Santos *et al*., 2015) and fish (Ferraro *et al*., 2004). It has a number of advantages over other genotoxic assays due to its ease of use and low cost as well as the requirement of only a small number of cells per sample (Tice *et al*., 2000). Therefore comet assay has been used in many studies to investigate DNA damage and repair by a variety of DNA-damaging agents.

In this study the comet assay was initially used to assess DNA damage in the MM cell line U266B1 and primary MSC following exposure to melphalan. Comet analysis was performed over a period of 72 hrs following drug exposure in order to measure both DNA damage and repair. Cells were seeded according to the model developed in chapter 3 with cells cultured independently and in a non-contact co-culture as well as looking at the possible bystander effect. After studying the effects of melphalan on primary MSC, the effects of this agent as well as the other chemotherapeutic agents used in this thesis were investigated with the stromal cell line HS5.

In all experiments primary MSC, HS5 and U266B1 cells exposed to H_2O_2 showed very high levels of DNA damage. The images captured after exposure to H_2O_2 showed a typical comet appearance with levels of DNA damage above 30% confirming that the technique is capable of detecting DNA damage. H_2O_2 is frequently used as a positive control in the comet assay as it is capable of creating oxidative DNA damage which can be detected by comet assay (Sondhi *et al*., 2010; Rank and Jensen, 2003). The extent of this damage is dependent on the concentration of H_2O_2 (Benhusein *et al*., 2010).

The comet assay is capable of detecting very small amounts of DNA damage (Olive and Banáth, 2006). Untreated cells that were cultured independently or in a co-culture with primary MSC or HS5 stromal cells showed no visually detectable damage to DNA with low tail intensities, at times below 5%.

Furthermore tail intensities remained low at subsequent time points indicating that there were also no delayed genotoxicity effects.

Timing of sampling after chemotherapeutic treatment may greatly impact on levels of genotoxic damage seen. Early genotoxic damage tends to arise from SSB, which are short-lived lesions and rapidly repaired (Brendler-Schwaab *et al.,* 2005). It is therefore recommended that comet assays employ at least one short incubation sampling time to ensure these lesions are not overlooked (Brendler-Schwaab *et al.,* 2005). Consequently, during this study cells were sampled immediately after drug exposure following a 1 hr incubation with chemotherapeutic agent. Moreover following this 1 hr incubation period, it is likely that some of the initial genotoxic effects seen are thus predominantly SSB.

The comet assay has been used to detect delayed DNA damage (e.g. apoptosis) or DNA repair following a recovery period (Olive *et al*., 1993). To determine the DNA damage recovery or delayed DNA damage to U266B1 and MSC/HS5 after chemotherapy, the comet assay was carried out immediately after treatment (1 hr), 16 (for melphalan) / 24 hr (for each other agent), 48 and 72 hrs after incubation of cells.

Melphalan was the only agent that caused significant DNA damage as measured by comet assay in U266B1 cells. Immediately after treatment of melphalan U266B1 cells reported an increase in DNA damage compared to the control. At 16 hrs comet tail levels were similar to the untreated control suggesting that damage had been repaired. However as it is known that melphalan causes DNA crosslinks (Spanswick *et al*., 2002) it was likely that the retardation in comet tails giving rise to low levels of DNA in the comet tail was caused by DNA crosslinks. DNA crosslinks are capable of stabilising DNA and preventing the DNA from migrating into measurable tail (Wu and Jones, 2012). Therefore, the effect of DNA crosslinks caused by melphalan treatment needs to be considered within this study, as this may have resulted in decreased DNA migration, potentially obscuring larger differences between untreated and treated cells. To confirm this was the case, DNA damage was

measured at 48 and 72 hrs. At each of these time points tail intensity increases due to the release of cross links.

Surprisingly no significant levels of DNA damage in primary MSC and HS5 stromal cells were reported at any time point. This could be due to the failure of these cells to influx the melphalan into the cell or could suggest that DNA damage generated by melphalan were either repaired or transformed into MN after cell division. As MN induction was evident in these cells (discussed later), it does suggest that MSC and HS5 are capable of in-fluxing the drug. As the comet assay only measures DNA fragmentation these observations indicate that DNA fragmentation does not occur within these cells following melphalan exposure. Future studies should look at the DNA methylation of MSC and HS5 stromal cells following melphalan exposure.

Immunomodulatory drugs, thalidomide and lenalidomide are efficacious in the treatment of MM and significantly prolong survival (Palumbo *et al*., 2012; Weber *et al*., 2003). However, the mechanisms of such effects of these agents have not been fully elucidated. Thalidomide has been reported to produce ROS, which causes oxidative damage to DNA (Aerbajinai *et al*., 2007) with very few reports indicating the DNA damaging properties of lenalidomide. Here the comet assay indicates that DNA damage did not occur in U266B1 and HS5 stromal cells following exposure to either of these agents. This however does not conclude that these agents are not genotoxic and other assays were conducted to confirm or dismiss genotoxicity in this setting.

The proteasome inhibitors bortezomib and carfilzomib have also improved the survival of MM patients (San Miguel *et al*., 2013; Lokhorst *et al*., 2008). Although they are not understood to target DNA directly, it was considered important for completeness to measure levels of DNA damage with these agents using the comet assay. Levels of DNA damage in U266B1 and HS5 following exposure to these agents was relative to the untreated control as expected. Future testing with the micronucleus assay however would reveal some potentially interesting findings.

Unfortunately, despite its advantages, the comet assay lacks standardization (Belpaeme *et al*., 1998) with differences in protocols employed amongst

laboratories (Olive and Banáth, 2006). Certain critical parameters in the comet assay protocol which may influence results gained, include agarose concentration, electrophoresis voltage, electrophoresis time, handling of cells during processing as well as duration of each incubation step. In this study it is important to mention that the thickness of the agarose gel on Gelbond film is similar to the thickness of the gel used in the traditional method on glass slides. Therefore there is no apparent difference in levels of DNA damage, between the two methods. Furthermore there is also no indication of different levels of DNA in the comet tail in different zones of the agarose gel on Gelbond film (Gutzkow *et al*., 2013). For each experiment, variation was minimised as far as possible; for example all paired/matched samples were run within the same batch to control for influences such as fluctuations in electrophoresis duration, which can result in the generation of small but significant increases in comet tails even in untreated cells (Hellman *et al.,* 1997). Furthermore comet analysis was performed using a semi-automated image-analysis system as recommended by the comet assay working group (Speit *et al*., 2015). In an ideal world, experiments would have been carried out using blind analysis to further reduce experimenter bias. As this was not always possible, slides were analysed in an orderly manner not excluding certain areas of the film.

As the comet assay predominantly detects SSB and DSB it is therefore necessary to use other genotoxicity assays that measure DNA damage such as the micronucleus assay to confirm the genotoxic potential of agents.

5.4.2 Detection of genotoxicity following chemotherapy using the micronucleus assay.

In the present work the micronucleus test was used to further investigate the DNA-damaging potential of each of the chemotherapeutic agents used in the study using the co-culture model. MN are small nuclear bodies present in the cytoplasm of cells formed by the exclusion of whole chromosomes or chromatin fragments during cell division (Norppa and Falck, 2003). Elevated frequency of MN could be related to an overall genetic instability (Balmus *et al*., 2015). Initially cytochalasin-B was used to block the cells at anaphase of

cell division. The cytokinesis-block micronucleus assay using cytochalasin-B restricts scoring of MN between once-divided cells that are accumulated and recognized by their BN appearance and undivided (mono-nucleated) cells (Fenech *et al*., 2003). Cytochalasin-B is an inhibitor of actin polymerization which blocks mitotic cytokinesis (Falck *et al*., 2002). It has, however been found to cause DNA fragmentation in a number of cell lines and gives rise to pycnotic nuclei (Nesslany and Marzin, 1999). Following exposure to cytochalasin-B a significant increase in apoptotic and necrotic U266B1 cells was observed. As such cytochalasin-B treatment was not used in this study. OECD test guidelines allow the use of protocols without cytokinesis block, provided there is evidence that the majority of cells analysed are likely to have undergone cell division (OECD guideline for the testing of chemicals, 2009). HS5 and U266B1 cells have a cell cycle of 50-60 hrs (ATCC and ECACC). With cells seeded 48 hrs prior to the first cell count at 1 hr post exposure it is likely that a high percentage of cells had undergone at least one cell division when cells were harvested at each time point. In order to confirm the cytotoxicity of each treatment the RICC was calculated for each cell line at each time point. Thalidomide and lenalidomide did not cause the RICC to drop below 50 % at any time point in any cell line. Proteasome inhibitors caused a drop in RICC below 50% in both HS5 and U266B1 cells at a number of time points post exposure and should therefore be interpreted with some caution.

Here a significant increase in MN was observed in all cultures exposed to melphalan. This finding correlated with a study by Mishra and Mishra, (2013) which found that melphalan induced MN in human lymphocytes in a dose dependant manner. Others have also reported an increase in MN in mammalian cells following exposure to melphalan (Efthimioua *et al*., 2007; Phousongphouang *et al*., 2000). However numbers of MN reduced in all cells when in co-culture demonstrating for the first time that a co-culture of MSC and U266B1 protects these cells from the DNA damaging effects of melphalan.

The mechanism of action of immunomodulatory agents thalidomide and lenalidomide have not been elucidated fully. A European Medicines Agency report on lenalidomide conducted a battery of genotoxic studies including a bacterial mutation test, a mouse lymphoma assay, a cultured human

peripheral blood lymphocyte test, a Syrian hamster embryo transformation assay and an *in vivo* rat micronucleus test and concluded there were no indications of genotoxicity in any of these assays (European Medicines Agency, 2008). It has also been widely reported that thalidomide is not mutagenic (Teo *et al*., 2000; Ashby *et al*., 1997). In this study the number of micronuclei in HS5 cells and U266B1 cells following exposure to lenalidomide were not significant in alone and co-cultures and concurs with previous reports on the genotoxic activity of these agents.

However, there was a significant rise in BN and multi-nucleated cells following exposure to these agents. BN cells arise following a failure of cytoplasmic division after nuclear division has taken place (Sagona and Stenmark, 2010) with further nuclear division leading to multi-nucleation. Thus multi-nuclei are cells containing 3 or more nuclei and one cytoplasm. Furthermore whilst bortezomib and carfilzomib also did not produce significant numbers of MN a rise in BN and multi-nucleated cells was observed. Following an extensive search of the literature, multi-nucleated cells have very rarely been documented. However some multi-nucleation occurs under physiological conditions in cells such as osteoclasts through the process of cell fusion (Jansen *et al*., 2012).

Although tri- and tetra- nucleated cells have been reported (Fenech, 2007) little emphasis has been placed on their impact on genotoxicity. Few studies have reported the effects of chemotherapy on multi-nucleation, although one study reported an increase in multi-nucleation following exposure to radiotherapy (Raj and Mahajan, 2011). To the best of knowledge, this current study is the first to document and evaluate a rise of these multi-nucleated cells following exposure to thalidomide, lenalidomide and bortezomib.

These results showed that an average of 2-4 multi-nucleated cells were present at each time point in U266B1 and HS5 untreated cells cultured independently and in co-culture. Multi-nucleation increased in U266B1 and HS5 cells at each time point when exposed to thalidomide, lenalidomide and bortezomib. The mechanism that gives rise to increased multi-nucleation is unclear. Some studies have reported abnormal cytokinesis with multi-

nucleation (Yu *et al*., 2015; Huang *et al*., 2008). A study carried out by Matsuoka *et al* (2010) using MDS-L cells found that 10 days after treatment of lenalidomide (10 μM), 50% of cells were multi-nucleated and suggest that mitosis is not affected but cytokinesis is disrupted. However a recent study by Rashid *et al*. (2015) reported that a derivative of thalidomide (5HPP-3) induced multipolarity and supressed microtubule dynamics by binding to the vinblastine binding site on tubulin in MCF-7 cells. Interestingly multi-nucleation following exposure to each of the agents decreased in cells that were co-cultured as opposed to exposure when cultured alone. This suggests that growth factors released from either cell type aid in the protection of genotoxicity.

Furthermore a bystander effect was seen in HS5 and U266B1 cells exposed to thalidomide. HS5 cells that were not exposed to thalidomide and cultured with U266B1 cells previously treated with thalidomide did not have significantly different numbers of multi-nuclei compared to HS5 cells that were exposed to drug and cultured with untreated U266B1. U266B1 cells that were not exposed to thalidomide and co-cultured with previously thalidomide treated HS5 cells had an increase in multi-nucleation. At 48 and 72 hrs there were higher numbers of multi-nucleated cells in cells that were not originally exposed to drug. These findings suggest a bystander effect occurs with thalidomide treatment. It is likely that thalidomide achieves this affect by increasing levels of intracellular ROS (Aerbajinai *et al*., 2007) with ROS an important mediator of bystander signalling (Widel *et al*., 2014).

The rise in multi-nucleation within HS5 and U266B1 could have far reaching consequences and may help explain the neurotoxic activity of thalidomide, lenalidomide and bortezomib (Argyriou *et al*., 2014; Delforge *et al*., 2010). Chemotherapy-induced PN is one of the main dose limiting toxicities of these medications with the exact pathophysiology unknown (Grisold *et al*., 2012). At present the literature suggests microtubule structures in the nerve axons are disrupted, however there are wide disparities in the proposed mechanism. Some suggest that the mode of action is via the inhibition of the vinblastine binding site (Rashid *et al*., 2015; Iguchi *et al*., 2008) which is a tubulin-polymer inhibitor. However others suggest that mode of action is through microtubule stabilisation such as that seen with taxol (Pandit *et al*., 2013; Li *et al*., 2006).

5.4.3 Genotoxic effects of immunomodulatory agents on TK6 lymphoblast cells following cell synchronisation

Following the findings of increased multi-nucleation following exposure to thalidomide and lenalidomide in HS5 and U266B1 cells, increased multi-nucleation was also identified in TK6 cells by Ms Jennifer Razik during her undergraduate study. TK6 are a human lymphoblast cell line that are p53 competent and regularly used during *in vitro* genotoxicity testing (Kimura *et al*., 2013; Sobol *et al*., 2012). To further investigate the effects of these agents, TK6 cells were synchronised before treatment with immunomodulatory agents. Synchronization involves the isolation of cells in specific cell cycle phases based on either physical properties or perturbation of cell cycle progression with biochemical constraints (Ma and Poon, 2011). Here a thymidine double block was used which allows the isolation of cells within a particular stage of the cell cycle, and enables a unique strategy for the analysis of molecular and structural events during cell division (Kabani *et al*., 2010).

The thymidine double block enabled the capturing of cells in M-phase and allowed the determination of the proportion of counted cells as BN and multi-nucleated. Numbers of multi-nucleated cells increased at each time point following exposure to each of the immunomodulatory agents suggesting that the damage is not repaired and the cells are capable of surviving 48 hrs post exposure. This may ultimately lead to genomic instability possibly via the downregulation of DNA damage response pathways (Broustas and Lieberman, 2014).

Here lenalidomide produced increased BN and multi-nucleation compared to the control in a time dependent manner, although numbers of multi-nucleated cells were not as high as those seen in thalidomide exposed TK6 cells. Lenalidomide is known to possess more potent anti-inflammatory and anti-angiogenic activities than thalidomide and holds a more favourable side-effect profile, in that the PN incidence is low, however therapy related cancer incidence is not (Delforge *et al*., 2010). The increase in multi-nucleation may be as a result of the many metabolites that are produced following thalidomide and lenalidomide treatment (Chen *et al*., 2012; Wells *et al*., 2010) and

therefore future studies should investigate the role of these metabolites on these cells.

A deep understanding of the disruption to microtubule structures during cell division will enable a greater understanding of the consequences of this rise in multi-nucleation. This could potentially explain both therapy related malignancy and teratogenic activity of these agents.

5.5 Conclusion

This study has shown a wide range of genotoxic effects in primary MSC, HS5 stromal cells and U266B1 following exposure to a number of chemotherapeutic agents used in MM. Investigation of DNA damage following chemotherapy found that melphalan was the only agent to induce significant levels of DNA damage as measured by the comet assay.

In vitro micronucleus assessment of these agents revealed some unexpected and potentially fundamental outcomes. The finding of a significant increase in multi-nucleated cells after exposure to immunomodulatory agents and the proteasome inhibitor bortezomib may be of fundamental importance and explain the PN seen in MM following exposure to these drugs - one of the most debilitating and treatment limiting side effects seen in MM. It is arguable that the impressive benefits of these agents outweigh the associated risks. However this study raises the urgent need to fully understand the mechanisms of these agents to define the appropriate conditions for their safe use in patients. Furthermore findings in this chapter reveal that both MM cells and HS5 protect each other from the genotoxic effects of some agents when in a non-contact co-culture.