Chapter 1

Introduction

1.1 Multiple Myeloma

Multiple myeloma (MM) is a haematological malignancy that originates from terminally differentiated B cells (plasma cells) and is characterised by their clonal expansion within the bone marrow (BM) leading to impaired haematopoiesis along with the presence of monoclonal immunoglobulin (M-protein or paraprotein) in the serum or urine (Jonsson et al., 2015; Munshi and Anderson 2013; Bruns et al., 2012). Overproduction of paraprotein in the blood is filtered into the urine, compromising renal function with patients also developing osteolytic bone lesions (Singhal and Mehta, 2006). Some publications suggest that these malignant plasma cells arise initially from the BM (Hoffbrand et al., 2005; Drexler and Matsou, 2000) while others believe that these cells circulate to and from extramedullary sites and then return to the BM where there is an appropriate environment for their expansion (Van Riet et al., 1998; Epstein, 1992). Treatments such as proteasome inhibitors and immunomodulatory drugs have significantly improved response and survival rates for patients with MM (San Miguel et al., 2013; Lokhorst et al., 2008). However, even with more therapeutic options available MM remains incurable, with patients developing advanced, relapsing disease, with drug resistance providing a major obstacle to further treatment (Gao et al., 2016; Yang and Lin, 2015).

1.1.2 Clinical features

Many of the clinical presentations involved in MM are non-specific with fatigue and severe bone pain the most common (Wahezi et al., 2015; Coleman et al., 2011). Bone disease is a major source of morbidity in MM and often causes persistent pain from lytic bone lesions and pathological fractures (Hameed et al., 2014). The bone disease is severe in MM due to the increase in osteoclastic bone destruction and inhibition of bone formation (discussed in section 1.4.2). Patients may also present with recurrent infection and anaemia due to BM infiltration of
plasma cells and progressive cytopenia (Terpos et al., 2015). Furthermore, patients often suffer with renal failure due to the excessive monoclonal light chains in the blood, and hypercalcaemia due to activation of osteoclasts with consequent demineralization of the bones, though all of these symptoms may not be seen in all patients (Dimopoulos et al., 2011).

Diagnosis is made by serum and urine electrophoresis or more commonly BM aspiration or biopsy. The minimum criterion for a diagnosis of MM is the detection of at least 10% abnormal plasma cells within a BM biopsy or a monoclonal protein level above 30 g/l in serum or above 1 g/24h urine collection (Kyle and Rajkumar, 2009). Plasma cell morphology from patients with MM can vary dramatically from small, mature, differentiated cells resembling typical plasma cells, to large, immature undifferentiated cells of 20-30 μm in diameter (Gertz and Greipp, 2004). Once the diagnosis is suspected, a radiographic skeletal survey is performed (figure 1.1).

**Figure 1.1 Radiographs from patients with MM.** A - Anterior posterior radiograph of right humerus. Radiograph indicating diffuse lytic lesions (top arrow) of varying sizes with old pathological fracture distal diaphysis (bottom arrow). Image taken with permission from: Healey et al., (2011). B - Radiograph of the skull demonstrating typical lytic lesions in MM. Image taken with permission from: Pienaar et al., (2008).
1.1.3 Epidemiology

According to the most recent survey by Cancer Research UK in 2013, MM was the 17th most common cancer in the UK accounting for around 2% of all new cases. In 2013, 3,142 (57%) men and 2,355 (43%) women were diagnosed with MM in the UK (Cancer Research UK, accessed June 2016). It is mainly a disease of the elderly and rarely affects people under the age of 40, with approximately 59% of cases diagnosed in people aged 70 years and over between 2011 and 2013, in the United Kingdom (figure 1.2) (Cancer Research UK, accessed June 2016). Worldwide MM is the second most common haematological malignancy with more than 114,000 new cases diagnosed in 2012, accounting for about 0.8% of all new cancers (Ferlay et al., 2014). Geographically, the frequency is very unevenly distributed in the world with the highest incidence in the industrialised regions of Australia / New Zealand, Europe and North America, and the lowest incidence rates in developing countries including Asia (Becker, 2011). Although not explained, this may be due to longer life expectancy and improved diagnostics in developed countries.

The incidence of MM has been recognized to vary by ethnicity with the highest incidence reported in blacks, with lowest incidence recorded in Asians (Waxman et al., 2010; Landgren and Weiss, 2009). In the United States of America (USA) incidence among African Americans is two-fold compared to white Americans (Alexander et al., 2007). According to the Surveillance, Epidemiology, and End Results (SEER) program of the National Cancer Institute (NCI) the mortality rate for MM from 2007 to 2011 was 7.7 (males) / 5.3 (females) per 100,000 population in blacks compared to 4.0 (males) / 2.5 (females) per 100,000 population of whites (SEER database, 2007-2011). The numbers of persons separated by race that were diagnosed with MM between 2009 and 2013 per 100,000 in the USA are presented in figure 1.3. The basis for these significant differences between races are complex and are likely to reflect social and economic disparities more than biological differences (Alexander et al., 2007).
Figure 1.2 Average number of new cases of MM per year and age-specific incidence rates of MM in males and females in the UK. MM is rarely diagnosed prior to age 40, after which the incidence increases rapidly until age 84 and then declines. Highest incidence rates recorded in males are in the 80-84 age bracket and the 85-89 age bracket in females. The average number of cases per year is higher in males compared to females up to ages 85-90 and 90+ (Image taken with permission from: Cancer Research UK, 2016).

<table>
<thead>
<tr>
<th>Male</th>
<th>Race</th>
<th>Female</th>
</tr>
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<tbody>
<tr>
<td>8.2</td>
<td>All</td>
<td>5.2</td>
</tr>
<tr>
<td>7.7</td>
<td>White</td>
<td>4.5</td>
</tr>
<tr>
<td>15.7</td>
<td>Black</td>
<td>11.5</td>
</tr>
<tr>
<td>4.7</td>
<td>Asian</td>
<td>3.0</td>
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Figure 1.3 Number of new cases of MM per 100,000 persons in the USA, by race and gender (Data sourced from Howlader et al., 2013).
1.1.4 Aetiology

As the understanding of the pathophysiology of MM progresses, along with the development of new therapies, the exact aetiology of MM remains unclear. Most studies have focused on long term environmental and occupational exposures, however many results are conflicting (Sergentanis et al., 2015; Glass et al., 2006). Studies have indicated a higher risk in those exposed to pesticides such as agricultural workers (Kachuri et al., 2013) as well as those exposed to organic solvents such as petroleum workers (Wong and Raabe, 1997) while others have not (Glass et al., 2006). Glass and colleagues (2006) found no evidence of an increased risk of MM associated with exposure to benzene. There have been cases reported of familial clustering of MM (Lynch et al., 2001). However, whether this is due to shared environmental factors or hereditary factors is unknown. No useful public health interventions have been identified which might reduce the overall incidence of this disease, or the individual risk of this disease. With the exception of well-established risk factors - male sex, older age, black ethnicity, - there is much uncertainty as to the aetiology of MM and further research is needed.

1.1.5 Prognosis and staging

Prognosis of MM is highly dependent on clinical and laboratory features. There are currently two MM staging systems used for predicting survival at diagnosis. The first clinical staging system for MM was developed by Durie and Salmon in 1975 using commonly available clinical parameters that predicted the prognosis for MM patients and helped to estimate tumour burden (Durie and Salmon, 1975). This system is based on a combination of clinical factors: amount of IgM protein, serum haemoglobin level, serum calcium level, number of lytic bone lesions on a skeletal radiographic survey and renal function (table 1.1; Angtuaco et al., 2004). In this system, survival times in months vary between 61 (stage 1), 54 (stage 2) and 30 (stage 3) (Handin et al., 2003).

The International Staging System was later described by the International Myeloma Foundation in 2005 (Greipp et al., 2005). This system is calculated by blood tests to measure serum beta-2 microglobulin ($\beta_2$M) and serum albumin,
classifying patients into risk groups with median survival times of 29, 44 and 62 months (Table 1.2). Research has demonstrated that together these two markers are very sensitive for determining the extent of the disease and are also useful for predicting survival (Greipp et al., 2005).

Table 1.1 The Durie-Salmon staging system for MM.

<table>
<thead>
<tr>
<th>Stage and Criteria</th>
<th>Value</th>
<th>Median Survival (Months)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>I: Low tumour burden</strong></td>
<td></td>
<td>61</td>
</tr>
<tr>
<td>Haemoglobin level</td>
<td>&gt;10g/dl</td>
<td></td>
</tr>
<tr>
<td>Serum calcium level</td>
<td>&lt;12 mg/dl (3 mmol/L)</td>
<td></td>
</tr>
<tr>
<td>Radiograph</td>
<td>No bone destruction or solitary plasmacytoma</td>
<td></td>
</tr>
<tr>
<td>Low paraprotein level</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum IgG</td>
<td>&lt;5 g/dl</td>
<td></td>
</tr>
<tr>
<td>Serum IgA</td>
<td>&lt;3 g/dl</td>
<td></td>
</tr>
<tr>
<td>Urine light chain</td>
<td>&lt;4 g/24 h</td>
<td></td>
</tr>
<tr>
<td><strong>II. Intermediate tumour burden</strong></td>
<td></td>
<td>54</td>
</tr>
<tr>
<td>All criteria</td>
<td>Between values for stage I and stage III</td>
<td></td>
</tr>
<tr>
<td><strong>III: High tumour burden</strong></td>
<td></td>
<td>30</td>
</tr>
<tr>
<td>Haemoglobin level</td>
<td>&lt;8.5 g/dl</td>
<td></td>
</tr>
<tr>
<td>Serum calcium level</td>
<td>&gt;12 mg/dl</td>
<td></td>
</tr>
<tr>
<td>Radiograph</td>
<td>More than two advanced lytic lesions</td>
<td></td>
</tr>
<tr>
<td>Low paraprotein level</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum IgG</td>
<td>&gt;7 g/dl</td>
<td></td>
</tr>
<tr>
<td>Serum IgA</td>
<td>&gt;5 g/dl</td>
<td></td>
</tr>
<tr>
<td>Urine light chain</td>
<td>&gt;12 g/24 h</td>
<td></td>
</tr>
<tr>
<td>Associated renal involvement</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A: serum creatine level</td>
<td>&lt;2 mg/dL</td>
<td></td>
</tr>
<tr>
<td>B: serum creatine level</td>
<td>&gt;2 mg/dL</td>
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</tbody>
</table>

The system divides MM into three stages based on haemoglobin, serum calcium and IgM levels. The purpose of such staging is to provide a summarised index of disease activity and severity, indicating prognostic significance and perhaps offering a guide to treatment. As renal failure is a severe complication of MM, patients are divided into 2 groups based on serum creatine levels A: <2 mg/dL, B: >2 mg/dL. (Data sourced from Greipp et al. 2005). [Abbreviations: g/dL, gram per decilitre; mg/dL, milligram per decilitre; mM/L, millimoles per litre; g/24hr, gram per 24 hrs].
Table 1.2 The International Staging System of MM.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Criteria</th>
<th>Median Survival (months)</th>
</tr>
</thead>
</table>
| I     | Serum $\beta_2$–microglobulin $< 3.5 \text{ mg/L}$  
Serum albumin $\geq 3.5 \text{ g/dL}$ | 62 |
| II    | Not stage I or III*                          | 44 |
| III   | Serum $\beta_2$–microglobulin $\geq 5.5 \text{ mg/L}$ | 29 |

*There are two categories for stage II: serum $\beta_2$–microglobulin $< 3.5 \text{ mg/L}$ but serum albumin $< 3.5 \text{ g/dL}$; or serum $\beta_2$ microglobulin 3.5 to $< 5.5 \text{ mg/L}$ irrespective of the serum albumin level.
** The normal healthy reference range for $\beta_2$ microglobulin is 1.20 – 2.4 mg/L.
*** The normal healthy reference range for serum albumin is 3.5 – 5.5 g/dl.

The system divides MM into three stages based on serum beta-2 microglobulin and serum albumin levels. (Data sourced from North Devon Healthcare Trust accessed 19/08/16; Nayak et al., 2011; Greipp et al. 2005). [Abbreviations: g/dL, gram per decilitre; mg/ll, milligram per litre].

1.2 Bone marrow microenvironment

Bone is a rigid connective tissue that provides support and protection for the vital organs of the body. Within the cancellous and long bone cavities the BM can be found of which there are two types; red and yellow. At birth, virtually all BM is red and over time is gradually replaced by yellow marrow (Gurevitch et al., 2007). Red marrow is comprised mostly of haematopoietic cells and is responsible for haematopoiesis (Pocock et al., 2013). The yellow marrow is not normally active but under times of severe stress such as severe blood loss it can be converted back to red marrow (Patton, 2015). Both types of BM consist of a pattern of blood vessels and nerves, which are encased by the endosteum membrane (Tortora and Derickson, 2006).

The BM microenvironment is a complex network of extracellular matrix (mainly collagen) which includes, osteoclasts, osteoblasts, lymphoid cells, fibroblasts and vascular endothelial cells which are in close contact with mesenchymal stem/stromal cells (MSC) (Kawano et al., 2015). Together these cells facilitate the survival, differentiation and proliferation of haematopoietic cells and non-haematopoietic stem cells such as MSC. This is achieved by a complex mix of
interactions with other cells from the same or different lineages via adhesion molecules or through the release of soluble factors (Wynn et al., 2004).

The highly organised microenvironment provides a safe haven for normal haematopoietic cells; however malignant haematopoietic and epithelial tumour cells that metastasize to bone may also take advantage, with the BM offering protection from chemotherapeutic agents (Cuiffo and Karnoub, 2012). In the last few years, MSC have been demonstrated to play important roles in tumour pathogenesis (Cuiffo and Karnoub, 2012; Reagan and Ghobrial, 2012) and are for this reason the subject of intense investigation and are discussed below.

1.2.1 Mesenchymal stem cells

MSC are a heterogeneous class of self-renewing, pluripotent cells that provide a supportive role within the BM and are known to play a vitally important role in supporting haematopoiesis (Li and Wu, 2011; Dazzi et al., 2006). MSC are not exclusive to the BM and have been isolated from adipose tissue (Dicker et al., 2005), placenta (Miao et al., 2006), umbilical cord and umbilical cord blood (Sibov et al., 2012). However, the exact proportion of MSC in these tissues is difficult to establish. Even in the BM where they are most abundant, their frequency is estimated to be as low as 0.001% - 0.1% of the total population of marrow nucleated cells (Pittenger et al., 1999).

Human MSC were first identified and isolated more than 40 years ago, in 1974 by Friedenstein and colleagues based on their high proliferative capacity and ability to adhere to plastic, giving rise to a layer of adherent spindle shaped cells in culture (Sarkar, 2009). Since their initial isolation from humans and with research focusing on their therapeutic potential, they have been successfully harvested from murine and canine models (Silva et al., 2005; Badoo et al., 2003). Numerous studies have shown that human MSC are capable of differentiating into a number of mesenchymal cell lineages most notably; adipocytes, chondrocytes and osteoblasts (Nakamizo et al., 2005; Jaiswal et al., 1997; Barry et al., 2001). More recently MSC have been reported to generate non-mesenchymal cells as well, such as epithelial cells, hepatocytes or even neuronal cells (Cattachio et al., 2013).
MSC can be isolated easily from BM and several other human tissues and using routine cell culture techniques can be expanded in vitro without loss of function or phenotype (Lucarelli et al., 2014; Romanov et al., 2005). MSC are selected from BM mononuclear cells by their adherence to tissue culture plastic within 24 - 48 hrs and can be visualised using light or phase contrast microscopy as an adherent monolayer of spindle shaped cells. In their undifferentiated state, standard culture conditions generally involve low glucose Dulbecco’s Modified Eagles’ Medium (DMEM) supplemented with 10% foetal bovine serum (FBS), penicillin-streptomycin (P/S), and L-glutamine with incubation at 37 °C in 5% CO₂ (Gregory et al., 2005). In an undifferentiated state MSC have a fibroblast-like appearance containing a small cell body with long, thin projections emanating from its centre (figure 1.4).

![Figure 1.4 Confluent bone marrow mesenchymal stem cells in culture. MSC are adherent cells that display a typically heterogeneous fibroblast-like morphology. Image x10 magnification.](image)

As no one universally accepted specific marker uniquely identifies MSC, it is therefore their immunophenotypic profile and characteristic morphology which identifies them, along with their extensive capacity for self-renewal while retaining the ability to differentiate along a number of mesenchymal lineages (Miao et al., 2006). In 2006, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) proposed minimal criteria to
characterize human MSC. This indicated that MSC must be positive for CD105, CD73, and CD90 and negative for haematopoietic markers CD45, CD34, CD14 or CD11b, CD79a, or CD19, and HLA-DR (Dominici et al., 2006). Furthermore, the criteria outlined that MSC must be adherent to plastic and must also be capable of differentiating into osteoblasts, adipocytes and chondroblasts in vitro (Dominici et al., 2006).

MSC are essential in forming the stroma of the BM; the stroma provides support to primitive haematopoietic cells, allowing them the ability to grow and differentiate within the BM (Fibbe and Noort, 2003). This support is achieved both by direct cell to cell interactions and/or by release of cytokines such as interleukin 6 (IL-6) and granulocyte colony-stimulating factor (G-CSF) (Majumdar et al., 2000; Oshima et al., 2005) and may also play an important role in the pathogenesis of MM, as these support mechanisms are harnessed by MM cells (Corre et al., 2007).

1.2.2 Effects of chemotherapy on MSC

Patients being treated for cancer will almost inevitably experience chemotherapy as part of their treatment regimen. As previously described, MSC are a critical component of the BM and therefore damage to MSC can have dramatic consequences on the BM microenvironment. Damage to MSC as a result of chemotherapy has previously been reported by Kemp et al., (2010) who found a reduction in MSC expansion and expression of CD44 after high dose chemotherapy (HDT) in vivo. Furthermore structural abnormalities and disruptions to the cell membrane morphology of MSC after exposure to the chemotherapeutic agents, cyclophosphamide and melphalan, have been observed (Kemp et al., 2011). Chemotherapy has also been shown to reduce the formation of colony forming unit-fibroblasts (CFU-F) and decrease the adhesive rate of MSC in colorectal cancer patients (Cao et al., 2008). Subsequent studies have demonstrated that chemotherapy induces DNA damage that may persist long term. Shortening of telomere length was observed following drug exposure and was evident after 28 days in culture (Buttiglieri et al., 2011). In contrast an investigation of MSC from previously chemotherapeutically treated patients
reported that MSC were resistant to chemotherapeutic substances, maintaining their characteristics \textit{in vitro} (Mueller \textit{et al.}, 2006).

However the full extent of the damage caused by chemotherapy on MSC has not been fully elucidated which may be of particular importance for those patients who receive a stem cell transplant. Whether this damage affects transplantation engraftment and haematopoietic recovery is not clear. Moreover, knowledge remains incomplete on the consequences of disease on the functionality of these cells. Whether diseases such as MM further exacerbate or protect from chemotherapeutic damage is unknown.

As MM cells are known to reside in the BM, this complex niche of cells including MSC provide crucial cues for MM growth, survival and provides chemo-resistance to current therapies (Basak \textit{et al.}, 2009). It has been documented that bone marrow – mesenchymal stem cells (BM-MSC) from MM patients differ from that of healthy individuals (Andre \textit{et al.}, 2013; Pevsner-Fischer \textit{et al.}, 2012; Corre \textit{et al.}, 2007). Despite these observations it remains unclear as to how MM impacts on functionality of MSC and whether MM cells protect MSC from chemotherapy.

\subsection*{1.2.3 Effects of MM cells on MSC}

It is now well accepted that MSC are important in tumour development in a range of haematopoietic malignancies, but whether MSC inhibit or promote cancer growth remains controversial (Clarke \textit{et al.}, 2015; Ljujic \textit{et al.}, 2013). MSC have been shown to display an avid tropism toward tumour cells in a wide range of malignancies including MM (Xu \textit{et al.}, 2012) and have been shown to promote growth and metastasis of colon cancer by enhancing angiogenesis, migration and invasion, and by inhibiting apoptosis of tumour cells (Shinagawa \textit{et al.}, 2010).

Research \textit{in vitro} has suggested that MSC from MM patients possess abnormal genomic, phenotypic, and functional properties, which might contribute to the progression of disease by providing a supportive niche that protects MM cells from spontaneous and drug-induced apoptosis (Giuliani \textit{et al.}, 2009). Zdzisinska and colleagues (2008) found that MSC from MM patients produced higher levels of IL-6, IL-10 and tumour necrosis factor alpha (TNF-$\alpha$) in response to the MM cell line RPMI 8226. In addition a recent study of MSC from MM patients found
that these cells release exosomes that modulate tumour growth in vivo (Roccaro et al., 2013). Other studies have documented that adhesion of MSC to MM cells provides the cancer cells with protection from chemotherapy (Kikuchi et al., 2015; Azab et al., 2009). Controversially MSC have also been shown to act directly on MM cells and inhibit their proliferation in vitro (Atsuta et al., 2013). Furthermore exogenously administered MSC were found to inhibit MM bone disease and tumour growth in an animal model (Li et al., 2012).

These studies reveal that MSC are at the centre of a complex cross talk of interactions that could drive MM cell growth and provide a sufficient niche to protect from chemotherapy. However, further clarification of the functionality of MSC in MM and following chemotherapy is required in order to exploit the distinctions between MSC in MM patients and healthy patients. To date, research has focused on MSC providing protection of MM cells to chemotherapy. However it is currently unknown whether MSC also become protected or are damaged when in the presence of MM cells and if their functionality is altered.

1.3 Adhesion molecules and cytokines

The cross-talk between cells located in the BM microenvironment and bone cells is tightly regulated. The “seed and soil” hypothesis was first introduced in the late 1800s by an English surgeon, Dr. Stephen Paget, who proposed a neoplastic growth (the seed, e.g., the MM cell) will proliferate in an environment (the soil, e.g., BM environment) that supports its replication. Since then, evidence has emerged demonstrating how components of the BM microenvironment are responsible for the proliferation of MM cells and in turn how this microenvironment aids in the survival of these cells (Wong et al., 2013; Hideshema et al., 2001).

The interactions of MM cells with the BM microenvironment is crucial to the pathogenesis of MM and involves various cytokines and adhesion molecules that provide positive and negative interactions between MM cells and MSC, as well as other cells of the BM microenvironment. These interactions activate a number of pathways in the MM cell including proliferative and anti-apoptotic signalling pathways (Hideshima et al., 2002 cited by Borrello, 2012) which stimulate
osteoclastogenesis (Michigami et al., 2000) and angiogenesis (Raimondo et al., 2000) (figure 1.5).

![Diagram of cell interactions in MM](image)

**Figure 1.5** Cell-cell interactions in MM. The communications between MM cell and MSC in the BM and the pathways and signalling molecules involved in the pathophysiology of MM (Image adapted from Andrews et al., 2013). (Abbreviations: VEGF-Vascular endothelial growth factor; IL1β - Interleukin 1 beta; IL-3 - Interleukin 3; IL-6 - Interleukin 6; IL-6R - Interleukin 6 receptor; JAK - Janus Kinase; STAT3 - signal transduction and activators of transcription 3; Ras - rat sarcoma; RAF/MEK/MAPK - Mitogen-activated protein kinases; M-CSF – macrophage colony stimulating factor; MIP1-α - macrophage inflammatory protein 1 alpha; TNFα - tumour necrosis factor alpha; RANKL - receptor activator of nuclear factor kappa-B ligand; NF-κB – nuclear factor kappa B).

Adhesion molecules are critically involved in the cellular interactions between MM cells and MSC. MM cells express an array of adhesion molecules including lymphocyte function associated antigen – 1 (LFA-1/CD18) (Ahsmann et al., 1992), very late antigen 4 (VLA-4/CD49d) (Jensen et al., 1993 cited by Damiano et al., 1999) and neural cell adhesion molecule (NCAM/CD56) (Camp et al., 1990). MSC express a vast array of adhesion molecules including CD106 (Yang
et al., 2013) and CD54 (Lee et al., 2009). These molecules bind to their cognate receptor/adhesion molecule on the surface of the MM since CD54 is a ligand for CD18 and CD106 is a ligand for CD49d and thus play an important role in the MM cell and marrow stromal cell interactions in vivo and in vitro (Tatsumi et al., 1996). Adhesion of the MM cell to MSC activates many pathways resulting in up-regulation of cell cycle regulating proteins and anti-apoptotic proteins in the MM cell (Yang et al., 2003).

The hallmark adhesion molecule of MM cells is CD138 known as syndecan-1. Syndecan-1 is a heparin sulphate proteoglycan that is expressed on the surface of mature plasma cells and also in a soluble form in the extracellular space of the BM (Yang et al., 2002). Much research has focused on its expression in MM with conflicting results. In 1998 a study by Dhodapkar and colleagues found that the addition of syndecan-1 to MM cell lines in vitro induced apoptosis and decreased osteoclastogenesis in BM cells. Whereas more recent studies have highlighted the detrimental effects of syndecan-1. An in vivo study using mice found that syndecan-1 was crucial to the growth, angiogenesis and metastasis of MM (Khotskaya et al., 2009). Furthermore, Ramani and Sanderson (2014), reported that chemotherapy stimulated the synthesis and shedding of syndecan-1, which may lead to the accumulation of high levels of syndecan-1 in the BM microenvironment and contribute to tumour progression and relapse.

Pathways that become activated as a result of the adhesion of MM cells to MSC include the phosphoinositol 3 kinase (PI3K)-protein kinase B (PkB/Akt), the IKK-α/NF-κB, Ras/Raf/MAPK and JAK/STAT3 and they can also be activated by numerous cytokines such as IL-6, IL-3, IL-10 and TNF-α of which both the MM and MSC secrete several (Ara and DeClerck, 2010; Chatterjee et al., 2002; Ogata et al., 1997). One of the major cytokines known to exert multiple effects within the BM microenvironment and has been implicated in MM is IL-6. IL-6 is a pleiotropic cytokine released as a result of pro- and anti-inflammatory stimuli and has an essential role in bone remodelling and MM progression (Scheller and Rose-John, 2006; Wallace et al., 2001).

The source of IL-6 has been the subject of much debate. In 1989 Klein and colleagues reported a paracrine and not autocrine regulation of IL-6 and that BM
stromal cells provided the major source of IL-6 within the BM microenvironment. Whereas other studies have shown that IL-6 is produced by MM cells themselves (autocrine) (Frassanito et al., 2001; Hata et al., 1993).

IL-6 achieves its survival effects by first binding to its cognate receptor on the surface of MM cells and then inducing the homodimerization of gp130 (figure 1.6). Following binding to the gp130 associated receptor IL-6R, the intracellular tyrosine kinase, janus kinase (JAK) is activated which phosphorylates and activates the STAT3 pathway (Mitsiades et al., 2006). Once the STAT3 is activated it translocates to the nucleus where it initiates transcription of IL-6 responsive genes. One such gene is BCL2L1 which encodes for the protein Bcl–XL which suppresses apoptotic death of haematopoietic cells (Catlett–Falcone et al., 1999). This protein works by inhibiting the release of pro-apoptotic molecules from the mitochondria (Arden and Betenbaugh, 2004). Catlett–Falcone and colleagues (1999) demonstrated that activated STAT3 contributes to the progression of MM by inhibiting its activation thus preventing the transcription of BCL2L1 and inducing apoptosis.

Similarly, IL-6 activates Ras and promotes its translocation to the plasma membrane where it activates Raf, mitogen activated protein kinase (MAPK) leading to increased proliferation of MM plasma cells (Hu et al., 2003). The function of this pathway is to transduce signals from the extracellular milieu to the cell nucleus where specific genes are activated for cell growth, division and differentiation. The Ras/Raf/Mitogen-activated protein kinase/ERK kinase (MEK)/extracellular-signal-regulated kinase (ERK) cascade couples signals from cell surface receptors to transcription factors, which regulate gene expression. Depending upon the stimulus and cell type, this pathway can transmit signals, which result in the prevention or induction of apoptosis or cell cycle progression (Chang et al., 2003). For example in MM cells constitutive ERK-signalling leads to the phosphorylation of the anti-apoptotic molecule, Mcl-1, decreasing its degradation, thereby promoting cell survival (Domina et al., 2004). This is further exacerbated by the suppression of a pro apoptotic protein known as Bim that binds to and neutralizes Mcl-1 expression which is targeted by ERK phosphorylation (Ley et al., 2003).
1.4 Multiple myeloma bone disease

1.4.1 Normal bone remodelling

In order to adapt to changes in growth, protect vital organs and maintain homeostasis, the human skeleton constantly undergoes remodelling throughout life. The process of bone remodelling is finely balanced, between bone resorption by osteoclasts and new bone formation by osteoblasts (Eriksen, 2010).

Osteoblasts are derived from MSC progenitors and are stimulated to differentiate during periods of active bone formation (Sims and Martin, 2014; Clarke, 2008).
The pathway by which osteoblast progenitors differentiate into mature osteoblasts is known as the canonical Wnt pathway. Wnts are cysteine-rich secreted glycoproteins which bind to the Frizzled receptor and low-density lipoprotein receptor-related protein (LRP-5/6) and induce the canonical Wnt pathway (Cong et al., 2004). The canonical pathway affects cellular functions by regulating β-catenin levels and via regulation of target genes elicits a variety of effects including induction of differentiation and proliferation of osteoblasts (Krishnan et al., 2006).

Osteoclasts are large multinucleated cells derived from haematopoietic cells in response to macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-b ligand (RANKL) and are responsible for bone resorption (Merrild et al., 2015; Boyce, 2013). RANKL is found on the surface of BM-MSC and osteoblasts (Ara and DeClerk, 2010). RANKL interacts with its cognate receptor RANK on the surface of immature osteoclasts causing their activation and differentiation into mature osteoclasts. In healthy individuals, this is a tightly regulated mechanism whereby osteoprotegerin (OPG), a decoy receptor, secreted by osteoblasts and MSC competes with RANK for binding to RANKL and thereby reducing osteoclastogenesis (Corallini et al., 2011; Simonet et al., 1997). OPG is a secreted factor that inhibits osteoclast development both in vitro and in vivo therefore favouring osteoblasts and enhancing bone formation (Simonet et al., 1997). The ratio of RANKL and OPG in patients with MM is markedly disturbed, with an increase in the expression of RANKL and decrease in the production of OPG (Roux and Mariette, 2004). Experiments using transgenic mice have highlighted the importance of the RANKL/RANK/OPG system in normal bone remodelling. Mice with a disrupted RANKL gene and those that overexpressed OPG had decreased osteoclast formation and developed an excessive accumulation of bone; a condition known as osteopetrosis (Kong et al., 1999). Mice deficient in OPG were shown to develop osteoporosis caused by enhanced osteoclast formation and function (Bucay et al., 1998; Mizuno et al., 1998).

During bone remodelling, bone is firstly resorbed by osteoclasts and osteoblast activity follows (figure 1.7). Osteoclasts attach to the surface of the bone and secrete H+ ions and proteinases, which demineralises bone, along with cathepsin
K secreted to degrade the matrix (Mandelin *et al*., 2006; Yamaza *et al*., 1998). Subsequently osteoblasts fill in these trenches by laying down new bone and ultimately become encased within the bone matrix giving rise to osteocytes (Dallas and Bonewald, 2010; Franz-Odendaal *et al*., 2006). If this fine balance of osteoclast bone resorption and osteoblast formation is unhinged it can lead to either excessive bone formation and osteopetrosis, or excessive bone destruction as seen in conditions such as osteoporosis and MM (Fattore *et al*., 2008; Hirayama *et al*., 2002).

**Figure 1.7 Mechanisms of bone remodelling in healthy individuals.** Normally bone homeostasis is a tightly regulated process whereby RANKL secreted by osteoblasts and BM-MSCs binds to the receptor RANK and stimulates the differentiation of osteoclasts. OPG acts as a neutralizing decoy receptor for RANKL and binds to RANKL inhibiting osteoclast formation. Osteoclasts travel across the surface of bone and release soluble factors that degrade the bone matrix and create lacunae that are filled with newly synthesised matrices by osteoblasts.

### 1.4.2 Bone remodelling in MM

In MM many different components interact to increase the bone resorption process and suppress bone formation resulting in prevailing bone destruction (Raje and Roodman, 2011). Much research has reported an increased osteoclast
activity and inhibition of osteoblasts, suggesting both cells are perturbed (Habibi et al., 2013; Farrugia et al., 2003; Choi et al., 2000). This bone destruction gives rise to osteolytic lesions that are detected in 70% to 80% of patients at diagnosis which leads to further complications including pathological fractures and hypercalcaemia (Terpos et al., 2013a).

Osteoclastogenesis is stimulated in MM by increasing RANKL expression and reducing the levels of OPG (Terpos et al., 2003). The mechanisms through which OPG levels are decreased have not been clearly defined yet, but a study by Standal et al., (2002) has shown that OPG is bound, internalized, and degraded by the MM cells through Syndecan-1. Others have demonstrated that adhesion of MM cells with BM-MSC and osteoblasts inhibits OPG mRNA levels and protein secretion by BM-MSC (Pearse et al., 2001).

Further to this, MM cells have also been shown to have an anti-apoptotic effect on osteoclasts by secreting high amounts of M-CSF (Dib et al., 2008). As a result, when RANKL binds to RANK in patients with MM there is a dramatic increase in bone resorption. Additionally, Abe and colleagues (2004) found that adhesion of MM cells to osteoclasts was partially dependant on IL-6 and osteopontin (a protein found in osteoblasts). Thus further highlighting the importance of IL-6 in MM.

In addition to increased osteoclast activity, the inhibition of osteoblast activity plays a critical role in the pathogenesis of MM (Giuliani et al., 2005). This decrease in osteoblast differentiation has been reported to be due to an increase in levels of cytokines such as IL-3 (Ehrlich et al., 2005), TNF-α (Gilbert et al., 2000) and MIP-1α (Vallet et al., 2011) in vitro.

The canonical Wnt/β-catenin pathway plays an important role in the survival of MM cells and in MM bone disease (Schmeel et al., 2013; Regard et al., 2012; Chen et al., 2001). The canonical Wnt/β-catenin pathway has been documented to control the expression of a number of genes such as CCND1 (Derksen et al., 2004). The CCND1 gene regulates physiological cell cycle progression from G1 to S phase and overexpression of this gene has been documented in MM patients (Specht et al., 2004; Malumbres and Barbacid, 2001). A study by Edwards and colleagues (2008) demonstrated that increasing Wnt signalling in the BM
microenvironment can prevent the development of osteolytic bone lesions. Lithium chloride treatment activated Wnt signalling in osteoblasts, inhibited MM bone disease, and decreased tumour burden in bone in an in vivo mouse model (Edwards et al., 2008).

Dickkopf-1 (Dkk-1) is an inhibitor of Wnt signalling that is secreted by MM cells and inhibits osteoblast differentiation (Zhou et al., 2013a; Qian et al., 2007). Elevated levels of Dkk-1 in the serum of MM patients were first observed by Tian et al., (2003) and were associated with the presence of bone lesions. Furthermore the severity of these bone lesions correlated with increased Dkk-1 levels (Kaiser et al., 2008). Finding that a soluble factor produced by MM cells suppresses osteoblast differentiation is significant, although it does not entirely explain why MM bone lesions do not heal even in patients in complete remission. It may be that a long-lasting change in the BM microenvironment inhibits the ability of osteoblast precursors to differentiate, notwithstanding the absence of MM cells.

As well as factors inhibiting the differentiation of osteoblasts, Silvestris et al. (2003) showed that osteoblasts from MM patients with severe skeletal involvement were functionally exhausted and undergo apoptosis in the presence of MM cells in vitro. A study by Karadag and colleagues (2000) reported that osteoblasts secrete high levels of IL-6 in response to MM cells thus promoting their proliferation and survival. All of these interactions result in a vicious cycle of increased bone resorption and increased tumour growth.

### 1.5 Treatment of MM

The first documented case of MM is of Sarah Newbury in 1844 who developed fatigue and severe bone pain, four years before her death (Solly, 1844). She later developed numerous fractures. She was administered infusions of orange peel, rhubarb pills and opiates but these failed to save her. She died on April 20, 1844, five days after being admitted to hospital (Solly, 1844). Autopsy showed that sections of the BM were replaced by a red substance along with a marked destruction and thinning of the bones (Solly, 1844). Treatment for MM has dramatically advanced since the first case described by Solly in 1844. In recent years novel targeted therapies have broadened and improved treatment for MM,
with MM patients living 10 years or longer post diagnosis and having a relatively normal quality of life (Ailawadhi, 2015). However, despite these advancements, MM remains an incurable disease.

As outlined above, the BM microenvironment promotes the survival and growth of malignant plasma cells, and this has led to the development of treatment strategies that inhibit certain interactions of the MM cell and BM microenvironment. In recent years the paradigm of MM therapy has undergone significant development and the introduction of novel therapies has resulted in improved survival (Catley et al., 2005).

1.5.1 Autologous stem cell transplantation

Autologous stem cell transplant (ASCT) has become a standard of care in newly diagnosed MM patients under 65 years of age. Usually, the procedure starts with chemotherapy (to reduce the tumour burden) prior to the collection of peripheral blood stem cells (Fung et al., 2010). High dose therapy (HDT) is then administered before the stem cells are replaced in order to increase the post HDT/ASCT complete response rate and to decrease the plasma cell BM infiltration to improve the quality of the graft (Mohty and Harousseau, 2014; Rodriguez et al., 2007). A number of studies have highlighted the importance of this treatment due to the increased complete response rates and prolonged survival compared to conventional chemotherapy (Moreau et al., 2015; Child et al., 2003; Attal et al., 1996). ASCT has also been shown to normalise the severe bone resorption and improve skeletal disease (Terpos et al., 2004). ASCT is traditionally preceded by HDT with alkylating agents such as melphalan (section 1.5.2) and followed with novel agents such as thalidomide (section 1.5.3), lenalidomide (section 1.5.4) and proteasome inhibitors (section 1.5.5). However many of these novel agents are now being used prior to ASCT such as thalidomide-dexamethasone, and bortezomib, doxorubicin, and dexamethasone regimens (Sonneveld et al., 2012; Tosi et al., 2010).

With MM predominately diagnosed in those over 70 years of age, a large proportion of patients are considered ineligible for ASCT due to safety concerns of HDT. However a recent study by Merz et al. (2014) found that ASCT with HDT
did not increase mortality in selected patients up to 75 years old. Further studies have also reported on the improved outcomes in these patients (Wildes et al., 2015; Bashir et al., 2012) and thus ASCT is beginning to provide a viable option to further improve the outcomes of elderly patients with MM. Furthermore, in an effort to improve outcomes, a second ASCT may be considered for patients who do not achieve a very good partial response after the first ASCT and has been shown to improve overall survival (Attal et al., 2003). However this remains controversial as this is not consistently observed, as others have reported no improvement in overall survival in patients who received a second ASCT (Cavo et al., 2007). For patients not eligible for ASCT or where it may not be required, a number of chemotherapeutic strategies are now available that are discussed further below.

1.5.2 Melphalan and Prednisone

Over the last 50 years the gold standard treatment for MM consisted of the oral alkylating agent melphalan (2-amino-3-[4-[bis-(2-chloroethyl)-amino]-phenyl]-propionic acid) in combination with prednisone (Alexanian et al., 1969). Melphalan was first synthesised in the 1950s as one of a series of nitrogen mustard derivatives (figure 1.8).

![Chemical structure of melphalan](image)

**Figure 1.8 Chemical structure of melphalan.** Melphalan \( (C_{13}H_{18}Cl_2N_2O_2) \) possesses two active alkyl sites that elicit its mechanism of action by adding an alkyl group \( (CnH2n+1) \) to DNA, most commonly at N7 position of guanine, leading to the formation of cross links thereby preventing DNA replication which ultimately leads to cell death.

Novel agents have recently been introduced, but this HDT is still used today as a pre-treatment for newly diagnosed MM patients before ASCT (Gertz and Dingli,
Conventionally, the upper age limit for this form of therapy has been around 65 years due to anticipated higher treatment-related toxicities in elderly patients (Ozaki and Shimizu, 2014). However a recent study has reported HDT melphalan prior to ASCT as safe in selected patients above 65 years old (Merz et al., 2014).

Melphalan is a phenylalanine derivative of nitrogen mustard that elicits its mechanism of action by the bifunctional alkylation of DNA (Hartley et al., 2002 cited by Spanswick et al., 2002). This is achieved by forming interstrand and intrastrand crosslinks with DNA, thereby inhibiting DNA synthesis and function (figure 1.9). Its most common site for alkylation of DNA is the N-7 position on the base guanine (Boysen et al., 2009). Alkylation of the base guanine may lead to mis-pairing with thymine or to DNA strand breakage. The second alkyl group on melphalan may undergo a similar reaction on another base on the opposite strand, thus forming a DNA crosslink. The cross helical base pairings cause strain and possible rupture of the double stranded DNA backbone (Hoskins, 2005).

![DNA crosslinks](image)

**Figure 1.9 Schematic image indicating the formation of DNA crosslinks as a result of melphalan treatment.** Crosslinking can occur either between two complementary strands of DNA (interstrand), or within a strand of DNA (intrastrand).

Melphalan is not cell cycle specific and enters the tumour cell by two distinct mechanisms; the L-amino acid system which transports leucine and the ASC system which transports alanine, cysteine and serine. Being a phenylalanine derivative its structure is similar to that of leucine and as a result, melphalan is
predominantly transported into the cell via the L-amino acid transport system (Uchino et al., 2002; Goldenberg et al., 1979).

Prednisone has an anti-inflammatory effect, thereby helping relieve pressure and pain in areas associated with the MM cell (Perry et al., 2012). It was first reported to induce remission in patients with refractory or relapsing MM by Salmon and colleagues in 1967. Alexanian et al. (1969) later found that when prednisone was combined with melphalan, there was a better rate of remission than administering melphalan alone.

In order to increase the chances of complete remission, anti-myeloma agents are used post-transplant as further supportive therapy. These include anti-angiogenic agents such as thalidomide and lenalidomide, bisphosphonates such as zoledronic acid (ZOL), the proteasome inhibitor bortezomib and most recently, heat shock protein inhibitors (Usmani and Chiosis, 2011; Terpos et al., 2009; Adams, 2003; Geitz et al., 1996). Each of these agents interacts with the BM microenvironment helping to reduce disease progression and improve patient survival.

1.5.3 Thalidomide

The immunomodulatory agent thalidomide (α-N-[phthalimido] glutarimide) is a synthetic glutamic acid derivative and has been used for the treatment of MM since 1997 and remains a key feature of treatment regimens today (Minarik et al., 2013; Kastritis and Dimopoulos, 2007). Thalidomide has a broad spectrum of activities in MM, yet its mechanism of action remains unclear. Its oral route of administration with minimal myelosuppressive effects makes it an attractive agent for maintenance therapy post ASCT. It was first synthesised in 1953 and was initially administered as a sedative and later to alleviate morning sickness during pregnancy (Yang et al., 2015). It was withdrawn from the UK in November 1961 and by 1962 from most of the world when it was reported that the drug produced severe, life threatening birth defects as a consequence of in utero exposure (Vargesson, 2015; Matthews and McCoy, 2003).

Despite its sombre past, thalidomide has proved successful in improving the management of MM in relapsed disease, as a first line therapy, as induction
before ASCT, as well as consolidation and maintenance regimens (Morgan et al., 2012; Palumbo et al., 2008; Rajkumar et al., 2006; Singhal et al., 1999). Thalidomide is known to have multiple actions including immunomodulatory, anti-inflammatory and anti-angiogenic properties (Kumar and Rajkumar, 2006).

In 1994 the ability of thalidomide to inhibit angiogenesis was first realised. A study by D’amato and colleagues (1994) demonstrated that thalidomide inhibited neovascularisation induced by basic fibroblast growth factor (bFGF) in the rabbit cornea micropocket assay. Furthermore, Kenyon et al. (1997) reported that thalidomide inhibited angiogenesis in a mouse model induced by bFGF and vascular endothelial growth factor (VEGF). Thalidomide may also inhibit adhesion of MM cells to marrow endothelial cells, as it can decrease the density of TNF-α-induced CD54, CD106, and CD62E and CD62L on the endothelial cells of human umbilical vein (Geitz et al., 1996).

Thalidomide monotherapy has achieved complete and partial response rates of below 30% in patients with relapsed or refractory MM (Glasmacher et al., 2006). To improve the efficacy of thalidomide it has often been combined with other chemotherapeutic agents. Thalidomide combined with the corticosteroid dexamethasone was shown to induce a high frequency of response, rapid onset of remission and low incidence of serious irreversible toxicity compared to thalidomide alone, in patients with previously untreated MM (Weber et al., 2003). Thalidomide and dexamethasone also produced higher response rates compared to dexamethasone alone (Rajkumar et al., 2008).

### 1.5.4 Lenalidomide

Lenalidomide is an immunomodulatory agent and is the first second generation analogue of thalidomide (Usnarska-Zubkiewicz et al., 2016). Its chemical structure is very similar to that of thalidomide with an amino group (NH2- ) at position 4 of the phthaloyl ring and removal of the carbonyl group (C=O) of the 4-amino–substituted phthaloyl ring (figure 1.10) (Dimopoulos et al., 2008). It was designed to have a more manageable safety profile than its parent compound and to provide more potent anti-inflammatory and immunomodulatory effects than thalidomide (Batoo and Hernandez-Ilizaliturri, 2011). Like thalidomide,
lenalidomide has anti-angiogenic properties and is a powerful inhibitor of TNF-α, as well as inhibiting the adhesion of MM cells to BM stromal cells and the release of growth and survival factors (Corral et al., 1999). It was approved in 2007 for the treatment of patients with MM (Usnarska-Zubkiewicz et al., 2016) and has been shown to be beneficial in relapsed as well as newly diagnosed disease (Weber et al., 2007).

A double blind trial compared the combination of melphalan-prednisone-lenalidomide followed by lenalidomide maintenance (MPR-R), melphalan-prednisone-lenalidomide (MPR) and melphalan-prednisone (MP) followed by placebo and reported a median progression-free survival significantly longer with MPR-R (31 months) than with MPR (14 months) or MP (13 months) with the greatest benefit observed in patients 65 to 75 years of age (Palumbo et al., 2012).

Figure 1.10 Chemical structure of thalidomide and lenalidomide. Structurally, lenalidomide has one amino and carbonyl group in its phthaloyl ring as compared with two carbonyl groups seen in the chemical structure of thalidomide (Kotla et al., 2009).
A phase 2 trial by Rajkumar et al. (2005) reported that lenalidomide combined with dexamethasone induced objective responses in over 90% of treated patients and complete or near complete responses in 38% of patients with newly diagnosed MM. Furthermore, a study by Attal et al. (2012) stated lenalidomide maintenance after transplantation significantly prolonged progression-free and event-free survival among patients.

1.5.5 Proteasome Inhibitors

1.5.5.1 The ubiquitin proteasome system

Effective protein degradation by the ubiquitin-proteasome pathway (UPS) is required in all cells in order to maintain cellular homeostasis (Herrmann et al., 2004). This complex process is performed by a number of enzymes that mark the protein for destruction by the 26S proteasome. The 26S proteasome complex is the central proteolytic machinery of the UPS that is present in the cytosol and nucleus of all eukaryotic cells (Wójcik and DeMartino, 2003). The UPS is responsible for the majority of intracellular protein turnover and elimination of miscoded, misfolded, damaged or abnormal proteins, as well as processing key regulatory proteins required for various cellular functions such as cell cycle progression and apoptosis (Jung et al., 2009; Adams, 2004). The 26S proteasome is made up of a 20S core catalytic complex containing three active enzymatic sites, including the chymotrypsin-like, trypsin-like, and caspase-like, activities with 19S regulatory subunits on each end (Jain et al., 2011; Adams, 2004). Inhibition of each of these active enzyme sites simultaneously, markedly reduces protein degradation with inhibition of the chymotrypsin-like activity of the proteasome contributing most to reduced proteolysis (Kisselev et al., 2006). This proteasome protein degradation pathway is outlined in figure 1.11.
Figure 1.11 Diagram of the structure and function the ubiquitin–proteasome protein degradation pathway. The UPS is initiated by the conjugation of ubiquitin to the substrate protein in an ATP-dependent manner. Proteins to be degraded are tagged with multiple units of ubiquitin. Polyubiquitinated proteins are then fed into the 26S proteasome and the ubiquitin molecules are recycled. The products are free small peptide fragments available as amino acids or used in antigen presentation (Vilchez et al., 2014; Lecker et al., 2006).

1.5.5.2 Bortezomib

Proteasome inhibition has been shown to have an important therapeutic impact in MM. The first proteasome inhibitor, bortezomib (VelcadeTM; PS-341) a boronic acid dipeptide, was a break-through MM treatment, targeting the 26S proteasome complex, and established proteasome inhibition as an effective therapeutic strategy (Chauhan et al., 2005) (figure 1.12). Bortezomib is a reversible inhibitor of the 26S proteasome and performs antitumour activity through multiple mechanisms, whereby it inhibits the production of cytokines such as IL-6 which suppresses MM cell growth as well as inhibiting angiogenesis (Roccaro et al., 2006). The apoptotic effect achieved by bortezomib is the result of blocking the degradation of the kinase IκB and blocking nuclear factor κB (NFκB) activation. NFκB signalling protects the cell from apoptosis by activating anti-apoptotic genes of the Bcl-2 family such as Bcl-XL and A1 (Karin and Lin, 2002). NFκB is a pro-survival transcription factor responsible for regulating the expression of a number of genes involved in cell proliferation and anti-apoptosis (Chang and Vancurova, 2013; Hoesel and Schmid, 2013). IκB is a natural inhibitor of NFκB, blocking its nuclear transcription. When IκB is activated it subsequently undergoes degradation by the proteasome (Mateos and San Miguel, 2007). Bortezomib prevents the degradation of IκB and subsequently the activation of NFκB and thus makes the MM cell more susceptible to apoptosis (Mannava et
Bortezomib has also been documented to significantly increase the expression of proteins involved in cell cycle progression such as p21 and p27 by preventing their degradation and thereby increasing cell cycle arrest (Nakamura et al., 2007). Furthermore, in 2001 Hideshema and colleagues confirmed that bortezomib can overcome resistance to conventional therapies reporting growth inhibition of MM cell lines that were resistant to melphalan, doxorubicin and dexamethasone.

A study by Richardson et al. (2005) reported that bortezomib was successful as a single agent in MM. A further trial by Richardson et al. (2007) of bortezomib in patients with relapsed MM following 1–3 prior treatments, reported an improved response rate, time to progression and overall survival compared to dexamethasone. Other studies have investigated the effects of bortezomib in combination with other chemotherapeutic agents. A trial by Cavo et al., (2010) assessed the addition of bortezomib to thalidomide and dexamethasone (VTD) versus thalidomide and dexamethasone (TD) in patients with previously untreated MM. The study showed higher complete response rates in those administered VTD compared to TD (31% vs. 11%). However side effects were significantly higher in those treated with VTD (56%) compared to TD (33%), with a higher occurrence of peripheral neuropathy (PN) in patients on VTD (10%) than in those on TD (2%) (Cavo et al., 2010). Other trials have investigated the effects of bortezomib with melphalan and prednisone (BMP) and have reported positive results (Azarm et al., 2012; Mateos et al., 2006). Mateos and colleagues (2006) documented an improvement in 3 categories with BMP compared to MP, response rate (89% versus 42%), event-free survival at 16 months (83% versus 51%), and survival at 16 months (90% versus 62%). Consequently, combination chemotherapy with bortezomib represents an attractive option for some MM patients.
Carfilzomib is an irreversible epoxyketone-based second-generation proteasome inhibitor that is chemically, structurally and mechanistically different from bortezomib and has been approved for treatment of relapsed and refractory MM (figure 1.11) (Kortuem and Stewart, 2013; Herndon et al., 2013). It has been demonstrated to provide sustained inhibition of the chymotrypsin-like activity within the proteasome and inducing apoptosis of MM cells (Jain et al., 2011). Compared to the first generation drug, bortezomib, it has also been documented to have a preferable safety profile as it has been associated with fewer cases of PN than bortezomib (Atrash et al., 2015; Siegel et al., 2013).

In 2012 a phase 2 study involving 129 bortezomib-naive patients with relapsed or refractory MM, were exposed to single agent carfilzomib and reported an
overall response rate of up to 52.2% (Vij et al., 2012). Other studies have looked at combining carfilzomib with other agents such as dexamethasone. In a phase 3 study, carfilzomib and dexamethasone was found to have a longer progression-free survival (18.7 months) compared to those treated with bortezomib and dexamethasone (9.4 months) in patients with relapsed or refractory MM (Dimopoulos et al., 2016).

1.5.6 Bisphophonates

Bisphophonates have been used in the management of a variety of skeletal disorders as well as haematological malignancies including MM (Berenson et al., 2002). Bisphophonates are pyrophosphate analogues and achieve their therapeutic role through inhibition of osteoclastic bone resorption by increasing apoptosis of osteoclasts thus improving bone strength and preventing skeletal related events of bone involvement in MM (Hiroi-Furuya et al., 1999). Bisphophonates are therefore an essential part of the management of MM, and are of particular clinical relevance in patients who have symptomatic bone loss (Terpos et al., 2013b). The bisphophonate, ZOL was shown to down-regulate the expression of MSC adhesion molecules (CD54, CD106, CD49d and CD40) which are involved in the cell-to-cell contact with MM cells (Corso et al., 2005). As a result, IL-6 production is decreased thereby also reducing MM proliferation.

1.5.7 Monoclonal Antibodies

Other novel agents used to combat the devastating bone destruction in patients with MM are monoclonal antibodies. One that has gained particular interest is denosumab. Denosumab has high affinity and specificity for RANKL by mimicking the effects of OPG, thereby binding to and neutralising RANKL, leading to inhibition of osteoclast function (Kostenuik, 2008). A recent study by Henry et al., (2011) compared denosumab to ZOL in preventing or delaying skeletal related events in patients with advanced cancer metastatic to bone or MM and found that it was non-inferior to ZOL.

Monoclonal antibodies have been found to complement currently available therapies. A study by Lonial et al. (2012), reported encouraging response rates in patients with relapsed or refractory MM with the combination of lenalidomide,
and low-dose dexamethasone with the monoclonal antibody, elotuzumab. This agent is known to target the signalling lymphocytic activation molecule-F7, a glycoprotein expressed on MM and natural killer cells but not on normal tissues that enables selective killing of MM cells with minimal effects on healthy tissue (Lonial et al., 2015; Hsi et al., 2008).

### 1.5.8 HSP90 Inhibitors

Heat shock protein 90 (Hsp90) is one of a family of heat shock proteins that are ATP-dependent molecular chaperones that become induced in response to cellular stress and regulates the stability and function of a diverse range of ‘client’ proteins involved in proliferation and apoptosis (Pearl et al., 2008). With Hsp90 involved in maintaining cellular homeostasis, and therefore cell survival, they have emerged as a potential target for the treatment of cancers including MM (Usmani and Chiosis, 2011; Powers and Workman, 2006). Hsp90 inhibition targets the ATP-binding pocket in the amino-terminal domain of Hsp90 causing a down-regulation of signalling pathways such as Akt (PI3K/Akt pathway), FAK (integrin pathway), Bcr-Abl (RAS/ERK pathway), and Apaf-1 (apoptosis) (Basso et al., 2002; Nimmanapalli et al., 2001; Pandey et al., 2000) which are all involved in MM cell survival.

Tanespimycin (17-allylamino-17-demethoxygeldanamycin, 17-AAG) was one of the first Hsp90 inhibitors developed and has been used in clinical trials as a single agent in patients with relapsed/refractory MM (Richardson et al., 2010). Other studies have combined Hsp90 inhibitors with proteasome inhibitors to great effect. A study by Ishii et al. (2012) using a mouse model, found that the combination of the Hsp90 inhibitor KW-2478 and bortezomib greatly reduced tumour burden in vivo and in vitro (Ishii et al., 2012).

### 1.5.9 Side effects of treatment

The length and frequency of each of these treatments may depend on the age of the patient, stage of cancer, underlying conditions and how the patient responds to them. The most common side effects for chemotherapy include, nausea, vomiting, anorexia, anaemia, diarrhoea, fatigue and hair loss (Veach et al., 2013; Lemieux et al., 2008). However with the introduction of novel agents in recent
years the most common and debilitating side effect of agents used to treat MM is PN (Kaley and DeAngelis, 2009; Wolf et al., 2008). PN is a dysfunction of the peripheral nerves (motor, sensory and autonomic) resulting in symptoms such as tingling and numbness of the hands and feet, loss of proprioception, temperature discrimination, impaired motor skills and pain (Mendoza et al., 2015; Wickham, 2007). PN is a significant dose-limiting toxicity of agents such as thalidomide and bortezomib and reduces the patient’s quality of life (Mohty et al., 2010; Corso et al., 2010). The pain experienced by PN may be so severe that treatment is delayed or stopped which may lead to disease progression (Park et al., 2013). Furthermore PN has been shown to be a long term side effect even after the cessation of treatment (Reyes-Gibby et al., 2009). Current knowledge of the mechanism underlying PN is very limited. A study by Cavaletti et al. (2007) noted that bortezomib induced pathological changes in Schwann cells and myelin of rats. Thalidomide’s PN effects are thought to arise from its inhibition of TNF-α and NF-κB (Mohty et al., 2010; Silberman and Lonial, 2008). Recently a study by Rashid et al. (2015) found that a thalidomide derivative altered microtubule structure which may suggest a possible mechanism for PN as microtubules are crucial to neuronal support.

1.6 Drug Resistance

Resistance to chemotherapy drugs is a major problem for the effective treatment of MM (Abdi et al., 2013; Matsui et al., 2008). While patients usually respond to initial chemotherapy, complete remissions are rare or relapse occurs and drug resistance subsequently appears with patients succumbing to refractory MM (Blade and Esteve, 2000). In many cases, cancer cells may become cross-resistant to a broad spectrum of structurally unrelated drugs after exposure to a single cytotoxic drug leading to a situation known as multiple drug resistance (Krishna and Mayer 2000; Covelli, 1999). Drug resistance can be either intrinsic or acquired. Intrinsic drug resistance, arises at the beginning of treatment with the cancer cells insensitive to chemotherapy treatment; whereas acquired resistance arises after the cancerous cells are initially sensitive to chemotherapy but later become progressively unresponsive to the same or different chemotherapeutic agents (Longley and Johnston, 2005; Goldie, 2001).
In order for the anti-tumour agent to exert its desired effect it must reach the plasma cell in a sufficient concentration. There are several mechanisms of drug resistance such as those that limit the amount of drug reaching the cancer cells and those affecting the tumour microenvironment. Reduced cellular drug accumulation may arise due to alterations in the uptake or efflux of the drug and could be responsible for the acquisition of resistance (Bellamy et al., 1991). Transporter proteins called ATP-dependent multidrug transporters associated with resistance are multi-drug resistance protein (MDR), multi-drug resistance associated protein (MRP1), lung resistance-related protein (LRP), and breast cancer resistance protein (BRCP) as these proteins play a major role in removing the drug from the cells (Schwarzenbach, 2002; Borst et al., 2000). Both MRP1 and LRP have been documented to be over-expressed following chemotherapy in MM (Nakagawa et al., 2006; Schwarzenbach, 2002). Nakagawa and colleagues (2006) reported an increased expression of MRP1 and LRP as inhibitor of apoptosis proteins in patients’ samples previously exposed to melphalan based regimens. As well as these proteins, intrinsic cell survival mechanisms including the overexpression of anti-apoptotic proteins (Bcl-XL), activation of NF-κB and Akt/MAPK signalling pathways lead to the malignant transformation of the plasma cell and ultimately drug resistance (Catley et al., 2005).

During recent years many studies have focused on the mechanisms underlying drug resistance in MM (Mutlu et al., 2015; Zhou et al., 2013b; Drain et al., 2011). The interplay between BM-MSC and MM cells performs a crucial role in the pathogenesis of disease. As previously mentioned the BM microenvironment contains many of the necessary components that encourage the progression and survival of MM cells (cytokines, such as IL-6 and physical interactions with BM-MSC). Previous work has demonstrated that adhesion of MM cells to BM stromal cells results in cell-adhesion mediated drug resistance (CAM-DR) (Xu et al., 2016; Liu et al., 2016; Ding and Shen, 2015). For instance CD49d has been documented to play an important role in CAM-DR through its interactions with Vascular cell adhesion protein 1 (VCAM-1/CD106) on the surface of BM-MSC (Sanz-rodriguez and Teixido, 2001; Damiano et al., 1999). Recently chemotherapeutic agents have been developed to target the adhesion of MM to
the BM stroma. For instance bortezomib has been demonstrated to suppress the expression of CD49d at the mRNA level and down-regulate CD49d, thus preventing its binding to CD106 and helping overcome CAM-DR. However despite this almost all patients inevitably develop drug resistance (Murray et al., 2014; Stessman et al., 2013).

Other studies have documented the role of cytokines and soluble factors in the rise of drug resistance in MM (Jones et al., 2016; Li et al., 2014). Hao et al. (2011a) reported that IL-6 promotes drug resistance in MM cells through suppressing miRNA-15a and miRNA-16 which regulate the growth and proliferation of MM cells. Recent reports have also recognised the involvement of exosomes (small membrane vesicles) in drug resistance in MM (Zhang et al., 2016; Guo and Guo, 2015). A study by Wang and colleagues (2014a) reported that BM-MSC secreted exosomes that increased MM cell viability, proliferation, and survival. Furthermore BM-MSC-derived exosomes inhibited the effects of bortezomib protecting MM cells from apoptosis.

Resistance to drugs in MM is an obvious clinical frustration that involves multiple complex mechanisms. Overcoming drug resistance is very complicated, and as novel therapies for MM are established that target these pathways, the challenge now is for pharmaceutical companies to address the multiple mechanisms of drug resistance. Furthermore, more attention needs to be focused on the crosstalk between MM cells and the BM microenvironment and in particular BM-MSC, which may shed light on understanding drug resistance in MM.

1.7 Genotoxic Effects Following Chemotherapeutic Insult

1.7.1 DNA damage

An essential requirement for all organisms is to maintain genomic integrity. The inability to do so can result in cell death or the stable inheritance of mutations that can have a devastating impact on cellular metabolism (Kousholt et al., 2012; Waters, 2006; Pastink et al., 2001). The complex series of cellular and molecular changes contributing to genotoxicity are mediated by a diversity of endogenous (metabolic processes) and exogenous (e.g. environmental agents) stimuli. These stimuli lead to the induction of DNA damage which plays a major role in
mutagenesis, carcinogenesis and ageing (Garinis et al., 2008; Freidberg et al., 2004). Cells incur DNA damage on a daily basis which needs to be repaired accurately and rapidly in order to maintain cell viability. It is estimated that every single human cell has to repair 10,000–20,000 DNA lesions every day (Lindahl, 1993 cited by Dianov and Hübscher, 2013). Potentially these lesions may cause mutations if not accurately repaired. DNA lesions can take a variety of forms, ranging from individual base damage, to strand breaks, DNA-DNA crosslinks and DNA protein crosslinks (Geacintov and Broyde, 2011).

Chemotherapeutic agents often target the DNA of cells (Cheung-Ong et al., 2013). However the full extent of their cellular mechanisms, which is essential to balance efficacy and toxicity, is often unclear. Genotoxicity testing provides indications of DNA damage which could lead to mutagenicity and is essential to the pharmaceutical industry, as registration of a pharmaceutical requires a comprehensive assessment of their genotoxic potential (Walmsley, 2007). Genotoxicity testing can be either in vivo or in vitro and is designed to detect compounds that induce genetic damage by various mechanisms. The Ames test and micronucleus assay are commonly used to detect for genotoxic damage and are regulatory tests for the pharmaceutical industry (Food and Drug Administration, 2012). The comet assay is still in development for regulatory testing but is used in research institutions as a measure of DNA damage (Burlinson, 2012).

DNA damage may be single strand breaks (SSB; discontinuities in one strand of the DNA double helix), double strand breaks (DSBs), adducts (addition of extra molecules to the DNA) and loss of nucleotide bases (Ribezzoa, et al., 2016; Jackson and Bartek, 2009). DSBs represent the most lethal form of DNA damage and are generated when the phosphate-sugar backbones of both DNA strands are broken at the same position or in sufficient proximity to allow physical dissociation of the double helix into two separate molecules (Aparicio et al., 2014). DSBs can be caused by exogenous stimuli such as ionizing radiation and chemotherapy such as etoposide (Vignard et al., 2013; Schonn et al., 2010) as well as endogenous sources such as reactive oxygen species (ROS) and failures in DNA replication (Klaunig et al., 2010; Jones and Petermann, 2012).
Chemotherapeutic agents used in the treatment of MM may directly or indirectly affect DNA integrity. As previously mentioned the most common alkylating agent used in the treatment of MM is melphalan, which causes both DNA mono-adducts and crosslinks, resulting in SSB or DSBs (Huang and Li, 2013). Although crosslinks only constitute a minor fraction of the DNA lesions introduced by melphalan, these DNA lesions are highly toxic for cells, impairing DNA transcription and replication, resulting in cell death if unrepaired (Deans and West, 2011; Balcome et al., 2004).

Immunomodulatory agents such as thalidomide can inflict DNA damage by increasing production of ROS (Aerbajinai et al., 2007). Under normal physiological conditions ROS have an important role in cellular homeostasis and are responsible for regulating signalling pathways and gene expression (Ray et al., 2012). Increased levels of ROS can induce oxidative DNA damage. A study by Hansen and colleagues (2002) reported thalidomide increased oxidative stress in rabbits. This finding along with others who have demonstrated thalidomide also induces DNA intercalation (Jonsson 1972, cited by Stephens et al., 2000) may explain thalidomide’s teratogenic effects which have not been fully elucidated.

Proteasome inhibitors have also been demonstrated to affect DNA as protein degradation regulates a wide range of cellular pathways including the DNA damage response (Takeshita et al., 2009). Bortezomib has been reported to suppress homologous recombination (HR), which plays a key role in DNA DSB repair (Murakawa et al., 2007). Furthermore bortezomib in combination with DNA damaging agents may enhance its efficacy through the suppression of HR-mediated DNA repair. The DNA repair pathways are discussed in further detail below.

1.7.2 DNA repair mechanisms

To avert the deleterious consequences of DNA damage, a number of DNA repair mechanisms have evolved including base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR) and recombinational repair. Each of these repair mechanisms in most cases restores the DNA sequences.
However, occasionally repair is erroneous, leading to point mutations, small and large insertions or deletions, and large scale rearrangements (Iyama and Wilson, 2013). DNA repair pathways have been investigated in MM due to the characteristic genome instability of the disease (Kassambara et al., 2014; Thyagarajana et al., 2013).

BER is the predominant DNA damage repair pathway that corrects multiple small base lesions that frequently occur in DNA e.g. alkyl or oxidative damage (Hickson, 1997). BER is normally defined as DNA repair initiated by a lesion-specific DNA glycosylases and is completed by either of two sub-pathways: short-patch BER; whereby only one nucleotide is replaced or long-patch BER; whereby 2–13 nucleotides are replaced (Almeida and Sobol, 2007).

NER is an important DNA repair mechanism necessary for repairing bulky DNA adducts and crosslinks caused by alkylating agents such as melphalan (Szalat et al., 2015). In NER, a helicase unwinds the DNA near the damage site and damage specific endonucleases cut at both sides of the damage, freeing an oligonucleotide fragment. Missing nucleotides are replaced by one or more DNA polymerases and repair is completed by DNA ligase (Cooper, 2000).

The MMR pathway recognises base-base and addition/deletion mismatches generated during DNA replication and there is an excision on the newly formed DNA strand containing this mismatch (Li, 2008). If there is a failure in MMR function it can result in an increase in spontaneous mutation rate (Klapacz et al., 2015). Moreover MMR status has been reported to influence the response of cells to chemotherapy (Papouli et al., 2004)

DSBs are among the most fatal DNA lesions because they disrupt the continuity of the DNA template essential for DNA replication and transcription. Repair of DSBs is complex and is achieved through two mechanisms; non-homologous end-joining (NHEJ) or homologous recombination (HR) (Lieber, 2010). The choice of repair is based on the phase of cell cycle and the abundance of repetitive DNA. NHEJ operates in all phases of the cell cycle whereas HR is most active in the G2/M phase (Takashima et al., 2009). NHEJ involves joining the broken strand ends of DNA by repair enzymes and is recognized as having a high potential for error (Chiruvella et al., 2013). HR is more specific than NHEJ and
involves the use of a complimentary DNA strand as a template to correctly synthesise new DNA, leading to the reconstitution of the original sequence (Li and Heyer, 2008).

If the DNA repair pathways are not properly regulated it can lead to further genetic instability and contribute to drug resistance in MM cells (Gourzones-Dmitriev et al., 2013). Over-activation of DNA repair pathways have been documented in MM (Herrero et al., 2015). Velangi et al. (2004) reported defects in MMR that increased in frequency during more active stages of the disease, suggesting a contributory role in disease progression. Another study found that single nucleotide polymorphisms genes involved in NER and BER were not associated with disease progression (Thyagarajan et al., 2013). Consequently, DNA repair pathways in MM are highly relevant to understanding the current spectrum of therapeutic agents used in this cancer. Moreover, as the treatment options for patients with MM are increasing, it is becoming important to identify the effects these agents have on DNA using appropriate culture systems.

**1.7.3 Therapy related malignancy**

Reported cases of secondary malignancy following MM date back to the 1960s where an association of MM with leukaemia was first reported (Nordenson, 1966 cited by Thomas et al., 2012). Since then clinical studies have reported an occurrence of acute myeloid leukaemia in patients treated with melphalan for MM (Oivanen et al., 2000; Cuzick et al., 1987). More recently studies have indicated an increased risk of developing a secondary haematological malignancy, with treatment strategies that included a combination of lenalidomide and melphalan (Palumbo et al., 2014). A study by Atall et al. (2012) reported 3.1% second primary malignancies in patients treated with lenalidomide compared to 1.2% in those treated with a placebo. Furthermore studies have also reported an increased risk of secondary malignancy in patients exposed to thalidomide (Krishnan et al., 2013). However to date there is not enough evidence to conclude that immunomodulatory agents definitively increase the risk of second cancers, but there is a cause for concern, although the overall benefit from these agents may outweigh this risk. With the rise of novel agents such as proteasome
inhibitors and HSP90 inhibitors there will be a substantial need to define their possible contribution to secondary malignancies.

1.8 Current culture systems

*In vitro* cell culture techniques have long been used to assess the effects of potential cytotoxic and genotoxic effects of drugs (Ekwall* et al.*, 1990). Cell lines have provided a useful tool to study the biological mechanisms involved in a wide range of diseases including MM (Ferreira* et al.*, 2013). A number of MM cell lines have been developed including U266B1, RPMI 8226 and Karpas 707 (Karpas* et al.*, 2001; Nilsson* et al.*, 1968). Other non-cancerous cell lines have also been developed including the BM stromal cell line HS5. The HS5 stromal cell line is an HPV-16 E6/E7 transformed cell line that represents functionally distinct components of the BM microenvironment (Roecklein and Torok-Storb, 1995). It has been documented in a number of BM studies, due to its ease of culture in comparison to primary MSC (Ramakrishnan* et al.*, 2013; Lwin* et al.*, 2007). This study began initially using primary MSC before continuing with the stromal cell line HS5.

The establishment of close connections governing MM cell growth and survival and their implications on BM-MSC underlines the need for appropriate models to study this complex interplay and evaluate the impact of chemotherapy on these cells. Traditional two-dimensional (2D) *in vitro* culture (static culture of single cells kept as a monolayer on flat, artificial surfaces) remain popular models for *in vitro* study, with recent directions in cancer research having increasingly used co-culture systems to study the interactions between different cell types (Goers* et al.*, 2014). Co-culture models have been developed in a number of cancer models to further understand the disease and cellular interactions including breast cancer (Heneweer* et al.*, 2005) and leukaemia (Civini* et al.*, 2013). These *in vitro* co-cultures offer a promising substitute to *in vivo* animal testing in drug research, offering a more representative human *in vivo*-like tissue model than animal models, with significant advantages in costs and time saving (Wu* et al.*, 2010). Furthermore co-culture models have been developed that allow chemotherapeutic agents to be metabolized *in vitro* (May* et al.*, 2012).
A number of MM studies have used inserts to separate cells of the BM microenvironment and MM cell lines to investigate their interactions (Markovina et al., 2010; Yaccoby et al., 2006; Giuliani et al., 2002). Yet many of these studies use different culture medium for the culture of the same cells. For instance a study by Ramasamy et al. (2012) cultured the MM cell line RPMI 8226 and stromal cell line HS5, in Dulbecco’s Modified Eagle Medium (DMEM). In contrast Slawinska-Brych et al. (2013) reported using Roswell Park Memorial Institute (RPMI) 1640 for the culture of RPMI 8226 and Mhyre et al. (2009) reported using RPMI 1640 for the culture of HS5 cells. Cell culture medium is not only important for the growth of cells *in vitro* but also providing the relevant substances for the differentiation of cells such as MSC. Furthermore co-cultures can differ in terms of cell density, culture volume, degree of separation between cultures and the time scale of co-culture (Goers et al., 2014). The standardization of culture conditions across laboratories is crucial to providing a suitable and relevant physiological model of the BM microenvironment in MM as well as in other conditions.

As well as co-culturing cells and exposing them to chemotherapy directly, another mechanism by which cells of the BM microenvironment might become damaged is via a bystander effect. For the purpose of this study a bystander effect refers to the cytotoxic or genotoxic damage of cells that have not directly been exposed to chemotherapy (Di et al., 2008; Djordjevic, 2000). Thus, the bystander effect is elicited indirectly by communication between cells via cell-to-cell gap junctions or through secreted, soluble factors into the local milieu (Sologuren et al., 2014). While the term bystander effect may cover a variety of distinct mechanisms, the common denominator is cellular interaction, either to increase or decrease viability of non-target cells (Djordjevic, 2000). Radiation induced bystander effects have been extensively studied (Mancuso et al., 2008; Morgan and Sowa, 2007; Belyakov et al., 2001). However relatively few studies have documented the effects of a chemotherapeutic bystander effect (Asur et al., 2009; Demidem et al., 2006). Furthermore the mechanisms of a bystander effect have not fully been elucidated, although both direct cell-cell contact and the presence of soluble factors have been demonstrated to be involved (Mitchell et al., 2004; Sowa-Resat and Morgan, 2004). There are two separate methods of invoking a bystander
effect either by the transfer of medium from treated cells to untreated cells or specifically treating one cell population and subsequently co-culturing with untreated cells. This study reports on the latter method. Moreover investigating the presence of a bystander effect might help to elucidate the functionality of MSC in the presence of MM cells and chemotherapy.

The development of suitable culture systems will lead to the better understanding of the interactions between cells of the BM microenvironment, the function of MSC in MM disease, DNA damage and potential bystander effects as a result of chemotherapy. These refined culture systems may lead to the improvement and development of chemotherapeutic agents used in MM as well as many other cancers.
1.9 Aims and Objectives

Hypothesis: BM-MSC altruistically protect MM from therapy and consequently become phenotypically and genetically compromised.

1. To develop and validate an *in vitro* model of the BM microenvironment in MM to enable the study of the effects of chemotherapeutic agents and MM cells on the BM stroma.
   - Culture conditions of both MM and HS5 cell lines as well as primary BM-MSC will be optimised in order to improve long term survival *in vitro*.
   - Proliferation and morphology of cells will be monitored in a range of culture media to find an optimum that would support both cell types.
   - A direct contact model will be attempted to be developed using cell culture inserts and validated using scanning electron microscopy.

2. To investigate the effects of chemotherapeutic treatment and MM cells on MSC/HS5 functional characteristics using the developed co-culture model.
   - Following establishment of the co-culture conditions, normal donor MSC and the stromal cell line HS5 will be exposed to clinically relevant chemotherapeutic agents used for MM therapy and their functionality will be tested with and without MM cell lines.
   - The cytotoxic effects of chemotherapy will be evaluated by investigation of the proliferative capacity, morphology and expression of important antigens on HS5, as well as evaluating their ability to differentiate and express certain cytokines.

3. To investigate the genotoxic effects of current chemotherapeutic treatments on MSC/HS5 and MM cell lines using the developed co-culture model.
   - Genotoxic damage to either MM cells or stroma caused by chemotherapeutic agents may be reduced or exacerbated as a result of a co-culture. Both MSC/HS5 and MM cells will be tested for levels of DNA damage using the *in vitro* comet and micronucleus assays at different time points after treatment.