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Resistance Mechanisms to Novel Therapies in Myeloma

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Abstract

The number of novel therapies for the treatment of multiple myeloma (MM) is rapidly increasing with proteasome inhibitors, immunomodulatory agents and monoclonal antibodies being the most well-known therapeutic classes whilst histone deacetylase inhibitors, selective inhibitors of nuclear export and CAR-T cells amongst others also being actively investigated. However, in parallel with the development and application of these novel myeloma therapies is the emergence of novel mechanisms of resistance, many of which remain elusive, particularly for more recently developed agents. Whilst resistance mechanisms have been best studied for proteasome inhibitors, particularly Bortezomib, class effects do not universally apply to all proteasome inhibitors, and within-class differences in efficacy, toxicity and resistance mechanisms have been observed. Immunomodulatory agents share the common cellular target cereblon and thus resistance patterns relate to cereblon expression and its pathway components. However, the cell surface antigens to which monoclonal antibodies are directed means these agents frequently exhibit unique within-class differences in clinical efficacy and resistance patterns. Despite the progressive biological elucidation of resistance mechanisms to these novel therapies, attempts to specifically exploit these processes lag considerably behind and until such approaches become available, resistance to these therapies will remain a concern.

Keywords: myeloma, novel therapy, drug resistance, proteasome inhibitor, immunomodulatory agent, monoclonal antibody

1. Introduction

There has recently been an explosion of novel agents for the treatment of MM that have dramatically improved overall response rates (ORR), progression-free survival (PFS) and overall

survival (OS) by targeting the malignant plasma cell and bone marrow microenvironment in unique ways. The main classes of novel agents are proteasome inhibitors, immunomodulatory agents and monoclonal antibodies, however several other classes of novel agents are emerging, including histone deacetylase inhibitors, BH3 mimetics, checkpoint inhibitors and selective inhibitors of nuclear export, as are alternative approaches, such as chimeric antigen receptor T-cells (CAR-T) with MM cell specificity. Whilst CAR-T technology in MM remains in pre-clinical and early clinical trial stages of development, this immunological approach is rapidly gaining momentum with several groups developing CAR-T cells for therapeutic use [1]. Despite these therapeutic advances, many MM patients develop disease relapse suggesting the development of drug resistance whilst some are primary refractory. In this chapter, for the three major classes of novel agents, we present a discussion on known biological mechanisms of resistance together with clinical trial efforts, if any, to overcome these. Of all therapeutic classes of novel agents, mechanisms of resistance to proteasome inhibitors have been studied in greatest detail and are the focus of this chapter.

2. Proteasome inhibitors

Plasma cells secrete immunoglobulin in response to infection and a range of other stimuli which requires folding in the endoplasmic reticulum (ER) lumen prior to secretion from the cell, resulting in a degree of ER stress due to misfolded protein [2]. ER stress is heightened in MM due to the high, sustained production of monoclonal immunoglobulin and a build-up of misfolded protein within the ER lumen. This ER stress activates three ER membrane stress sensors, protein kinase RNA-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6) in a homeostatic process termed the Unfolded Protein Response (UPR) [2]. Activation of the UPR results in a global reduction in protein translation and the upregulation of ER chaperones and folding machinery to cope with the misfolded protein load, thereby rectifying the high ER stress levels that initiated the process. However, high sustained levels of ER stress can overwhelm the corrective capacity of the UPR which turns from a pro-survival, homeostatic mechanism to one that commits the MM cell to apoptosis. By inhibiting the 26S proteasome and preventing the degradation of misfolded proteins, proteasome inhibitors induce ER stress and a terminal UPR [2]. However, there are other mechanisms through which these agents exert their activity. Indeed, proteasome inhibitors are able to modulate a diverse array of cell signalling pathways whilst rendering the microenvironment less supportive of MM cell growth [3]. Perhaps due to the significant clinical impact the first-in-class proteasome inhibitor Bortezomib has made, resistance mechanisms to this agent have been studied in greatest detail compared to other proteasome inhibitors (**Table 1** and **Figure 1A**).

2.1. The ubiquitin-proteasome pathway

The ubiquitination and proteasome degradation pathway is a multistep enzymatic cascade in eukaryotes through which the cell removes excess and misfolded proteins and regulates

Resistance type	Resistance mediator(s)	Resistance mechanism
Proteasome inhibitors		
Mutations	*PSMB5* mutations encoding the β5 proteasome subunit	Impaired ability of proteasome inhibitors to bind to the catalytically active *N*-terminal threonine in proteasome subunits
Aberrant expression of ubiquitin-proteasome pathway components	β5 and other proteasome subunits	Increased or decreased numbers of binding sites for proteasome inhibitors, altering their ability to inhibit proteolysis
Activation of the aggresome-autophagy pathway	HDAC6 and autophagic machinery	Sequestration of toxic proteins in aggresomes and their removal by autophagy-mediated degradation
Heat shock protein induction	Grp78, Hsp90 and other family members including Hsp70 and Hsp8	Increased protein chaperoning resulting in greater ability to deal with misfolded and other toxic proteins
Drug efflux activity	P-glycoprotein and other ATP-binding cassette (ABC) superfamily members	Cellular efflux of proteasome inhibitors thereby reducing their ability to interact with proteasome subunits and other intracellular processes
Antioxidant response pathway induction	Over-expression of nuclear factor, erythroid 2 like 2 (*NFE2L2*)	Assists proteasome assembly by inducing expression of proteasome maturation protein (POMP)
Plasma cell differentiation	Reduced expression of Xbp1	Correlates with reduced immunoglobulin synthesis and ER stress/proteasome load therefore reduced sensitivity to proteasome inhibitors
Bone marrow microenvironment	Adhesion molecules on MM cells and bone marrow stromal cells (e.g. CD138, CD44, VCAM-1, LFA-1, MUC-1, ICAM-1 etc.)	Microenvironmental protection from proteasome inhibitors and other anti-MM therapies by increased MM cell migration, homing and adhesion to the bone marrow and activation of survival and proliferative intracellular signalling pathways
Survival signalling pathways	IL-6, VEGF, HGF, c-MET, NF-κB, PI3K/AKT, IGF-1/IGF-1R, tight junction protein 1 (TJP1) and EGFR/JAK/STAT signalling	Proliferation and cell survival signalling reducing the efficacy of proteasome inhibitors. Increased angiogenesis and MM cell migration. Induction of EGFR/JAK/STAT signalling associated with increased expression of proteasome subunits
Immunomodulatory agents		
Cereblon pathway abnormalities	Reduced cereblon expression	Less available target for IMiD binding
	Cereblon and other pathway component mutations	Reduced ability for IMiDs to bind to cereblon and other pathway components
Ras/Raf pathway activation	KRAS G12D and BRAF V600E mutations	Ras/Raf pathway activating mutations result in MM cell proliferation and resistance to IMiDs
Adhesion to bone marrow stroma	CD44 (Wnt/β-catenin signalling)	Greater adhesion to bone marrow stromal cells protecting MM cells from IMiDs
Monoclonal antibodies		
Target antigen expression	Reduced expression of CD38, SLAM7 and other cell surface proteins	Less available target for mAb binding through various mechanisms including trogocytosis

Resistance type	Resistance mediator(s)	Resistance mechanism
Resistance to complement-dependent cytotoxicity (CDC)	Increased expression of CD46, CD55 and CD59	Reduced ability for mAbs to activate CDC
Soluble antigen	Extracellular CD38 and SLAM7	Extracellular binding of mAbs to target antigen resulting in reduced mAb binding to cell surface antigen
Development of neutralising antibodies	Anti-mAb antibodies	Host derived anti-mAb antibodies neutralise therapeutic mAbs before reaching their cellular targets

Table 1. Mechanisms of resistance to the main classes of novel agents for multiple myeloma [143].

cellular processes including cell proliferation and survival [4]. The process involves the conjugation of ubiquitin via a lysine residue at position 48. Proteins tagged with lysine 48-linked chains of ubiquitin are marked for degradation in the proteasome enzyme complex [5, 6]. Eukaryotic cells contain the 26S proteasome which consists of a 20S core particle that is bound to two 19S regulatory particles [7, 8]. The 19S regulatory particle is responsible for substrate recognition, deubiquitination, unfolding and translocation into the 20S core particle which contains the active sites that hydrolyze substrate peptide bonds [9]. The 20S core particle is composed of four rings that are composed of seven α (α1–α7) subunits or seven β subunits (β1–β7), that are stacked in a specific order ($\alpha_7\beta_7\beta_7\alpha_7$). These rings generate three interconnected chambers: two outer chambers that are formed by the adjacent α and β rings and a catalytic chamber that is formed by the two adjacent β rings. Only the β1, β2 and β5 subunits are catalytically active proteases [10, 11]. Near the β subunit's active site lies a substrate specificity pocket which binds to 10 amino acid stretches in the substrate that flank the peptide bond that is cleaved and thereby determines the cleaving preferences of each β subunit [12, 13]. In particular, the β1 subunit has caspase-like activity (cleaving after acidic residues), β2 exhibits trypsin-like activity (cleaving after basic residues), and β5 has chymotrypsin-like activity (cleaving after hydrophobic residues) [14, 15].

Proteins that are targeted for proteasomal degradation must cross the 19S regulatory subunit in order to reach the proteolytic 20S core where they are degraded into peptides that vary from 3 to 25 amino acids in length [16, 17]. Each substrate is cleaved in multiple locations without release of partially hydrolyzed substrates from the core particle and the mechanism of degradation is conserved for all catalytically active β subunits [16, 18]. In eukaryotes, the 20S core particle components can change in response to biological stimuli. For example, stimulation of cells with interferon gamma induces the expression of all three catalytically active β subunits. These subunits, along with a unique 11S regulatory particle, form a complex called the immunoproteasome which is involved in generating peptides for presentation to major histocompatibility complex class I molecules, but also has classic proteolytic activity [19–21]. Increased expression of the immunoproteasome complex has been reported in MM, where it may represent the predominant form of the proteasome [22–25]. It is also noteworthy that relapsed MM may be associated with lower levels of the immunoproteasome and increased levels of the constitutive proteasome [25].
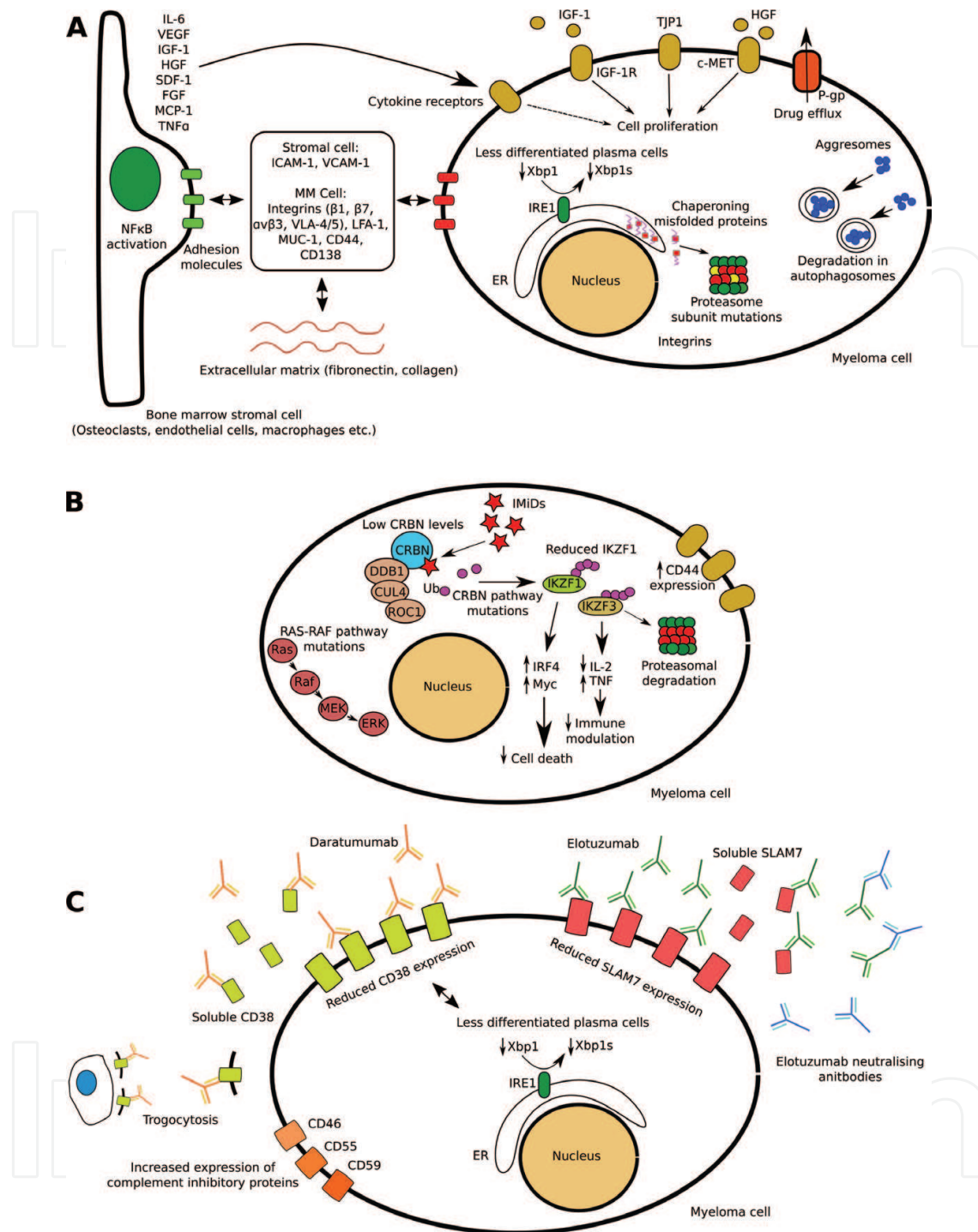
Figure 1. Known resistance mechanisms for the main classes of novel MM therapies [143]. (A) Proteasome inhibitors. (B) Immunomodulatory agents (IMiDs). (C) Monoclonal antibodies. See text for details. c-MET, hepatocyte growth factor receptor; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; IGF-1, insulin-like growth factor; IL-6, Interleukin-6; ICAM-1, intercellular adhesion molecule-1; LFA-1, lymphocyte function-associated antigen-1; MCP-1, monocyte chemotactic protein 1; MUC-1, Mucin-1 antigen; P-gp, P-glycoprotein; SDF-1, stromal cell-derived factor; TNFα, tumour necrosis factor alpha; VCAM-1, vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor; VLA-4/5, very late antigen 4/5; Xpb1, X-box binding protein 1; ZO-1, Zonula occludens-1; CRBN, cereblon; Cul4, Cullin-4; DDB1, DNA damage-binding protein 1; Erk, extracellular signal-regulated kinases; IKZF, IKAROS family zinc finger; IL-2, Interleukin-2; IRF4, interferon regulatory factor 4; Mek, mitogen-activated protein kinase kinase; MYC, MYC proto-oncogene; Raf, rapidly accelerated fibrosarcoma; ROC1, regulator of cullins 1; Ub, ubiquitin; SLAM7, signalling lymphocytic activation molecule family member 7.

2.2. Proteasome inhibitors used to treat myeloma

Proteasome inhibitors are potent anti-MM agents which inhibit one or more proteolytic subunits of the 20S proteasome. Their efficacy is attributed to a number of factors including inhibition of NF-κB signalling, although this has recently come under question, induction of ER stress with the activation of a terminal UPR, and modification of the bone marrow microenvironment, amongst others [26, 27]. Several generations of proteasome inhibitors have been developed with Bortezomib, Carfilzomib and Ixazomib approved for clinical use in a number of countries. The proteasome inhibitors differ in their relative selectivity for β catalytic subunits, and half-life and reversibility of β subunit inhibition, that translates into differential anti-MM efficacy and toxicity profiles [26]. Thus, the individual proteasome inhibitors demonstrate significant within-class pharmacokinetic and pharmacodynamic variation and resistance to one proteasome inhibitor does not necessarily suggest resistance to another.

The first-in-class proteasome inhibitor Bortezomib (N-acyl-pseudo dipeptidyl boronic acid) is a dipeptide that binds *reversibly* to the chymotrypsin-like β5 subunit of the catalytic chamber of the 20S proteasome and to a lesser extent the β1 and β2 subunits [26]. Attempts to improve the efficacy and toxicity profiles of Bortezomib resulted in the development of the epoxyketone Carfilzomib, an *irreversible* 20S proteasome inhibitor that preferentially binds to and inhibits the chymotrypsin-like β5 subunit with demonstrated activity in Bortezomib-resistant MM patients (ASPIRE trial) [28, 29]. Like Bortezomib, Ixazomib is a *reversible* peptide boronate 20S proteasome inhibitor of the chymotrypsin-like β5 subunit also with activity in Bortezomib-resistant MM as demonstrated in the TOURMALINE phase III trial [30]. Unlike Bortezomib, however, Ixazomib is orally bioavailable and found to induce less toxicity in patients, possibly due to its much shorter β5 subunit dissociation half-life [31].

2.2.1. Later-generation proteasome inhibitors

There are ongoing attempts to expand and improve the repertoire of proteasome inhibitors. Marizomib *irreversibly* inhibits the three proteolytic sites of the 20S proteasome and pre-clinical studies have shown efficacy in Bortezomib-resistant MM cells [32]. A phase I study evaluating Marizomib, Pomalidomide and Dexamethasone in heavily pre-treated patients with relapsed/refractory MM demonstrated an impressive ORR of 53% and clinical benefit rate of 64% [33–35]. This new proteasome inhibitor will likely be examined in more advanced clinical trials in the near future, not only for its ability to re-sensitise patients to proteasome inhibition but for its activity in MM involving the central nervous system. Oprozomib is structurally similar to Carfilzomib with the advantage of being orally administered and has demonstrated pre-clinical efficacy in Bortezomib-resistant MM cells [36]. Whilst there are no clinical trial results at this time in relapsed/refractory MM, several early phase studies are currently active, including a phase Ib/II study of Oprozomib in combination with Dexamethasone (NCT01832727) and with Pomalidomide and Dexamethasone (NCT01999335 and NCT02939183).

2.3. Mechanisms of resistance to proteasome inhibitors

2.3.1. Mutations and aberrant expression of ubiquitin-proteasome pathway components

2.3.1.1. Pre-clinical/clinical findings

Several point mutations in proteasome subunits that render them insensitive to Bortezomib inhibition have been identified. A single point mutation in the Bortezomib binding pocket of the β5 subunit (*PSMB5* gene) resulting in substitution of Ala49 with Thr (A49T) was described in Bortezomib-resistant human myelomonocytic THP1 cells, generated by culturing cells in escalating concentrations of Bortezomib [37]. This mutation was also detected in Bortezomib-resistant Jurkat cells, as were other mutations including A49V and the combination of A49T with A50V [38, 39]. However, despite the A49T β5 subunit mutation being detected in Bortezomib-resistant KMS-11 and OPM-2 human MM cell lines, no such β5 mutations were detected in Bortezomib-resistant RPMI-8226 MM cells, suggesting other mechanisms of resistance were at play [40, 41]. There have been a number of other β5 mutations identified in pre-clinical studies which affect Bortezomib binding and until recently, no mutations in *PSMB5* have been detected in either newly-diagnosed MM patients or those with relapsed and/or refractory disease [42, 43]. However, the first report of *PSMB5* mutations in a patient resistant to Bortezomib has renewed interest in this area although the clinical significance of these mutations is yet to be determined [44].

Significantly increased protein expression of the β5 subunit and only modest increases in β1 and β2 subunits were observed in Bortezomib-resistant THP1 cells which were reversible upon withdrawal of Bortezomib from cell cultures [37]. Over-expression of β subunits has also been detected in some MM cell lines, as well as those of some other haematologic malignancies, however, studies in MM suggest that the induction of these proteins is at most modest with minimal contribution to resistance [45]. Furthermore, free β5 subunits are catalytically inactive by themselves and cannot generally bind proteasome inhibitors unless assembled into functional proteasomes [46]. The expression levels of tight junction protein 1 (TJP1/ZO-1) were shown to be strongly associated with Bortezomib sensitivity with the downstream mechanism being suppression of EGFR signalling, which decreased the levels of proteasome subunit synthesis in at STAT3-dependent manner [47]. High TJP1 expression in patient MM cells was associated with a significantly higher chance of responding to Bortezomib and a longer duration of response [47].

2.3.2. Activation of the aggresome-autophagy pathway

2.3.2.1. Pre-clinical/clinical findings

Cytosolic small protein aggregates form when misfolded proteins accumulate, which are then transported towards the microtubule organising centre into a structure called the aggresome. Acetylation of α-tubulin, which is reversed by histone deacetylase 6 (HDAC6), modulates the structure and function of the microtubule, thus playing a pivotal role in the movement of misfolded protein aggregates to the aggresome [48]. Cells that lack HDAC6 were found to be

defective in the removal of protein aggregates and are not able to form large aggresomes [49]. Autophagy is predominantly a pro-survival homeostatic process whereby double-membrane vesicles known as autophagosomes sequester cytosolic proteins, including aggresomes, followed by fusion with lysosomes for degradation. Thus, misfolded proteins can be degraded via the ubiquitin-proteasome and/or aggresome-autophagy pathways and simultaneous blockade of both by combining Bortezomib and the HDAC inhibitor Panobinostat, respectively, showed synergistic anti-MM activity in pre-clinical models [50]. By inhibiting the proteasome, Bortezomib results in an increase in aggresome formation and also induction of autophagy, the latter a likely compensatory mechanism to eliminate misfolded proteins and other substrates of the ubiquitin-proteasome system which could be involved in resistance to proteasome inhibitors [51]. Thus, clinical studies combining a proteasome inhibitor with HDAC and/or autophagy inhibition have a sound biological basis for overcoming resistance to proteasome inhibitors.

2.3.2.2. Clinical studies to circumvent resistance

A large phase III study demonstrated a superior PFS when Panobinostat was combined with Bortezomib and Dexamethasone over Bortezomib and Dexamethasone alone in relapsed/refractory MM patients, leading FDA approval of Panobinostat in 2015 [52]. Despite this, no differences in OS or ORR were evident although the proportion of patients achieving a complete response (CR) was higher with Panobinostat. Given the activity of Carfilzomib in Bortezomib-resistant MM, early clinical studies are ongoing examining the combination of Panobinostat and Carfilzomib in relapsed/refractory MM and are expected to yield favourable results (NCT01496118). With regard to autophagy, a phase II trial evaluating the combination of Bortezomib and the autophagy inhibitor Chloroquine in patients with relapsed and/or refractory MM, supported by the finding of synergistic MM cell death in the pre-clinical setting, showed a clinical benefit rate of 40%, further cementing the role of the aggresome-autophagy pathway in proteasome inhibitor-resistant MM [53].

2.3.3. Heat shock protein induction

2.3.3.1. Pre-clinical/clinical findings

The heat shock response is part of the cell repair machinery that maintains homeostasis under stressful conditions such as infection, inflammation, starvation, hypoxia, and exposure to toxins, which is carried out by heat shock proteins (HSPs) [54]. HSPs assist in protein folding and preventing undesirable protein aggregation [54]. Blockade of proteasome-mediated protein degradation leads to the induction of HSPs and related chaperones, which have been shown to confer resistance to proteasome inhibitors [55]. Two well characterised HSPs in this setting are Grp78 (*HSPA5*; also known as Binding immunoglobulin protein, BiP) and Hsp90 (*HSP90AA1*).

Grp78 resides in the ER lumen where it is bound to the luminal domains of the three ER stress protein sensors, ATF6, PERK and IRE1 [2]. Upon accumulation of misfolded proteins in the ER, Grp78 (1) detaches from ATF6, PERK and IRE1 enabling activation of the homeostatic UPR and (2) chaperones the misfolded proteins for degradation by the 20S proteasome [2].

In MM, Grp78 was reported to play a role in resistance to proteasome inhibitors, and MM cells surviving proteasome inhibitor treatment showed increased Grp78 expression, which further increased with progressive disease [56]. However, this was not corroborated by others who could not demonstrate any significant differences in Grp78 expression in bone marrow plasma cells obtained from patients with MGUS, newly-diagnosed MM or relapsed/refractory MM [57]. Inhibition of Grp78 can induce MM cell death and pharmacological inhibition of Grp78 with Metformin, genetic ablation or mutational inactivation followed by Bortezomib treatment led to the accumulation of aggresomes, impaired autophagy and enhancement of the anti-MM effects of Bortezomib [58].

Hsp90 expression also increases with the accumulation of misfolded proteins in the ER lumen and has been investigated as a potential target to enhance the efficacy of Bortezomib [59]. Hsp90 was found to stabilise Grp78 at the post-transcriptional level, and treatment of Bortezomib-resistant mantle cell lymphoma cells with the Hsp90 inhibitor IPI-504 together with Grp78 knockdown led to synergistic cell death when combined with Bortezomib [60]. Other HSPs have also been shown to confer resistance to Bortezomib, including Hsp70 and small heat shock protein B8 (Hsp8) in MM and Hsp27 in lymphoma [61, 62].

2.3.3.2. Clinical studies to circumvent resistance

No advanced clinical trials employing Grp78 modulation in MM patients have been undertaken, although a study using an anti-Grp78 monoclonal antibody induced a PR in a heavily pre-treated patient when combined with Bortezomib and Lenalidomide [63]. Whilst early clinical trials have identified safe dose ranges for Hsp90 inhibitors, which have been tested either alone or in combination with Bortezomib and Dexamethasone in relapsed/refractory MM, results have been disappointing and to date no agents have progressed beyond the phase I/II stage [64].

2.3.4. Drug efflux

2.3.4.1. Pre-clinical/clinical findings

The efflux of drugs by members of the ATP-Binding Cassette (ABC) superfamily is a well-established mechanism by which tumours are able to acquire therapeutic resistance [65]. Whilst the multi-drug efflux transporter MDR1/P-glycoprotein (P-pg/ABCB1) has been shown to correlate with MM relapse and drug resistance [66, 67], its role in Bortezomib resistance has been controversial and it is generally thought that Bortezomib is a poor substrate [68]. P-gp was rarely detected in newly diagnosed MM patients [67], however, over-expression was associated with disease relapse and drug resistance, specifically to Vincristine, Doxorubicin, Etoposide and glucocorticoids [66, 67, 69]. Carfilzomib, on the other hand, is a *bona fide* P-gp substrate and patients treated with Carfilzomib show increased P-gp expression [70]. Upregulation of P-gp in MM cells confers resistance to Carfilzomib [71]. To date, there are no studies that relate P-gp to drug resistance to Ixazomib. Whilst Carfilzomib resistance in MM can be reversed *in vitro* by P-gp inhibition, for example using Verapamil or Vismodegib [72], this has not yet translated into clinical trials.

2.3.5. Antioxidant response pathway induction

2.3.5.1. Pre-clinical/clinical findings

Elevated levels of antioxidant-related pathway genes have been associated with drug resistance in other tumours, including resistance to Bortezomib in patients with mantle cell lymphoma [73]. Bortezomib resistance-related gene expression signatures revealed enrichment for Nuclear Factor, Erythroid 2 Like 2 (*NFE2L2*) which is activated as part of an antioxidant response pathway [74]. The downstream *NFE2L2* gene target *POMP* encodes the proteasome maturation protein proteassemblin, a chaperone responsible for the assembly of active proteasome particles from inactive precursor subunits [75]. Recently, *POMP* was found to be a mediator of the Bortezomib-resistant phenotype in MM cells [75], however, these findings have not been applied clinically.

2.3.6. Plasma cell differentiation

2.3.6.1. Pre-clinical/clinical findings

The transcription factor Xbp-1, a downstream component of the IRE1 arm of the UPR, is required for the differentiation of B-cells into plasma cells and more recently has been shown to be associated with Bortezomib sensitivity [76, 77]. Patient-derived bone marrow MM cells can be subdivided into populations based on their expression of Xbp-1, with plasma cells expressing low or absent Xbp-1 enriched in the bone marrow of patients who have relapsed after Bortezomib therapy or who have progressive disease [76]. These low or absent Xbp-1 expressing plasma cells were less differentiated with lower levels of immunoglobulin synthesis, reduced ER stress and less proteasome load. Conversely, at MM diagnosis, the majority of bone marrow plasma cells expressed higher Xbp-1 levels, conferring sensitivity to Bortezomib, although subpopulations of plasma cells with lower levels could be detected [76]. It is hypothesised that these subpopulations of plasma cells with low Xbp-1 expression are responsible for eventual relapse after induction therapy [76]. Interestingly, these findings would suggest that patients who are resistant to proteasome inhibitors should have non-secretory MM, however, only a small minority of these patients have this disease phenotype. To date, the degree of plasma cell differentiation has not been considered in clinical trials.

2.3.7. Bone marrow microenvironment and survival signalling pathways

2.3.7.1. Pre-clinical/clinical findings

The bone marrow microenvironment (BMME) includes (1) the non-cellular compartment formed by extracellular matrix (ECM) proteins (laminin, fibronectin and collagen) and soluble factors (cytokines, chemokines and growth factors) and (2) a cellular compartment comprising haemopoietic cells and non-haemopoietic cells (fibroblasts, osteoblasts, osteoclasts, endothelial cells, endothelial progenitor cells, pericytes, mesenchymal stem cells and mesenchymal stromal cells) which support MM cell survival and growth [78]. The interaction between ECM proteins and bone marrow stromal cells (BMSCs) with MM

cells plays a crucial role in MM pathogenesis and drug resistance by secreting growth factors, cytokines and extracellular vesicles (exosomes) and by the expression of adhesion proteins [78].

Various soluble factors have been shown to confer resistance to Bortezomib and other therapeutic agents in MM. IL-6 enhances vascular endothelial growth factor (VEGF) secretion promoting angiogenesis which plays a role in MM cell migration [79]. Whilst Bortezomib can inhibit IL-6 and VEGF production, secretion of IL-6 by stromal cells and MM cells leads to Bortezomib resistance [80]. Hepatocyte growth factor (HGF) is upregulated during MM progression, enhancing the expression of its receptor, c-MET [81]. This signalling pathway is constitutively activated in MM cells and endothelial cells from patients with relapsed/refractory MM and mediates drug resistance [82]. Accordingly, an inhibitory effect on endothelial cells obtained from patients refractory to Bortezomib or Lenalidomide was demonstrated using the c-MET inhibitor SU11274 alone or in combination with Bortezomib or Lenalidomide, resulting in downregulation of angiogenic activity [83].

Constitutive activation of pro-survival signalling pathways (e.g. NF-κB and AKT) has been reported to reduce the sensitivity of MM cells to Bortezomib [84]. Insulin-like growth factor (IGF-1) is produced by plasma cells and is present in the BM microenvironment, where it promotes proliferation and drug resistance in MM cells through activation of MAPK and PI3K/AKT signalling cascades [85]. Over-expression of IGF-1/IGF-1R pathway components has been shown to be a potential mechanism for resistance to proteasome inhibitors with blockade of downstream IGF-1 effectors able to resensitise MM cell lines to Bortezomib [86]. Studies evaluating compounds that affect the IGF-1/IGF-1R interaction are ongoing with OSI-906, a small molecule inhibitor of IGF-1R, able to resensitise MM cells to Bortezomib [86]. A downstream target of IGF-1, AKT, increases in expression in response to proteasome inhibitors in pre-clinical MM studies and an early phase clinical trial suggests that AKT inhibition might overcome resistance to Bortezomib [87]. As previously discussed, reduced expression of tight junction protein 1 (TJP1/ZO-1) and downstream activation of EGFR signalling are strongly correlated with Bortezomib resistance [47].

Interactions between MM cells and the BM stroma and/or ECM components provide a mechanism whereby MM cells are protected from the cytotoxic effects of anti-MM therapies. Such interactions include those mediated by adhesion molecules of the integrin family, Syndecan-1 (CD138), CD44, vascular cell adhesion molecule-1 (VCAM-1), lymphocyte function-associated antigen-1 (LFA-1), Mucin-1 antigen (MUC-1) and intercellular adhesion molecule-1 (ICAM-1) [88]. The adhesion of MM cells to stromal cells triggers IL-6 secretion, NF-κB activation in stromal cells and activation of signalling pathways that result in MM cell survival and proliferation [88]. Such effects are seen with integrin β7 which increases MM cell adhesion, migration and homing into bone marrow and reduces Melphalan and Bortezomib-induced apoptosis [89]. Similar MM-promoting effects have been reported for the stromal cell-derived factor (SDF-1)/CXCR4 axis, however, clinical translation has not ensued [90].

Other important mechanisms of BMME-induced drug resistance are emerging. BMSCs can modulate certain miRNAs in MM cells [91]. The expression of miR-27a is associated with

Bortezomib resistance in MM patients [91] whilst suppression of miR-15a and -16 by BMSCs was shown to be responsible for the protection of MM cells from Bortezomib-induced apoptosis [91]. miR-29 acts as a tumour suppressor miRNA and is downregulated in patient MM cells and in MM cell lines with acquired resistance to Bortezomib, Carfilzomib and Ixazomib [91]. Finally, exosomes mediate local cell-cell signalling by transferring mRNAs, miRNAs and proteins. It has been shown that exosomes derived from BMSCs inhibited Bortezomib-induced cell death to protect MM cells from apoptosis [92].

2.3.7.2. Clinical studies to circumvent resistance

In a phase II study, the anti-IL-6 antibody Siltuximab was administered with Dexamethasone to patients with relapsed and/or refractory MM [93]. Although no responses to Siltuximab alone were observed, the addition of Dexamethasone resulted in ORR, PFS and OS of 23%, 3.7 months and 20.4 months, respectively. Despite these findings, this strategy has not progressed further. The c-MET inhibitor Tivantinib was examined as a single agent in a phase II study in relapsed/refractory MM patients [94]. Overall, 36% of patients showed stable disease as their best response with the authors concluding that Tivantinib did not show promise for unselected relapsed/refractory MM patients, however, the fact that a significant proportion did show disease stability suggests combining c-MET inhibition with other anti-MM therapy could be explored. There are a small number of phase I studies employing a monoclonal anti-IGF-1R antibody alone or in combination with Bortezomib in relapsed/refractory MM, however, the authors of one study conclude that due to low response rates, even in combination with Bortezomib, further development is not justified [95]. Note should be made that patient recruitment into this study was not performed based on evaluation of IGF-1R expression on patient MM cells. No small molecule inhibitors of IGF-1R have so far been tested clinically. A phase I clinical trial in relapsed/refractory MM patients suggests that AKT inhibition with Afuresertib might overcome resistance to Bortezomib [87]. In this study, the ORR was 8.8%, however, despite these potentially promising results in heavily pre-treated patients, more advanced clinical trials have not been undertaken.

3. Immunomodulatory agents

The immunomodulatory drugs (IMiDs), Thalidomide, Lenalidomide and Pomalidomide have also made a major impact in the management of MM. Despite a checkered history in the 1950s and 1960s due to teratogenicity, Thalidomide has high anti-MM activity and has been incorporated into many treatment regimens. The second generation IMiD Lenalidomide and third generation IMiD Pomalidomide represent sequential improvements in efficacy and toxicity profiles with demonstrable activity in patients who have developed resistance to an earlier generation IMiD [96]. With regard to Lenalidomide, the MM-009 [97] and MM-010 [98] phase III trials demonstrated the superiority of Lenalidomide and Dexamethasone over Dexamethasone in relapsed/refractory MM patients whilst the pivotal MM-003 study [99] demonstrated the efficacy of Pomalidomide and Dexamethasone in MM patients who were refractory to both Bortezomib and Lenalidomide.

The anti-MM effects of IMiDs are related to their binding to the E3 ubiquitin ligase cereblon (CRBN) and subsequent ubiquitination and degradation of two B-cell transcription factors, Ikaros (IKZF1) and Aiolos (IKZF3) [96]. A landmark study identified CRBN as a primary target in Thalidomide teratogenicity, further demonstrating that Thalidomide binds to CRBN, disrupting the function of the E3 ubiquitin ligase complex, ultimately leading to the downregulation of fibroblast growth factor genes and the teratogenic effects associated with Thalidomide [100]. Subsequently, it was shown that the anti-MM efficacy of IMiDs is directly related to CRBN expression.

3.1. Mechanisms of resistance to immunomodulatory agents

3.1.1. Pre-clinical/clinical findings

Resistance mechanisms to IMiDs have been elucidated to a far lesser extent than have those for proteasome inhibitors (**Table 1** and **Figure 1B**) and mostly hinge on the presence of functional CRBN in MM cells [100]. MM patients exposed to and thought to be resistant to Lenalidomide had lower CRBN levels compared to paired samples before and after therapy [101]. Subsequently, it was shown that high expression of CRBN is associated with a favourable response to Thalidomide and Lenalidomide in newly-diagnosed MM patients [102, 103] and no IMiD response occurred in patients with very low CRBN levels [104]. Moreover, in MM patients refractory to Pomalidomide, CRBN levels predicted for differences in PFS (3 versus 8.9 months) and OS (9.1 versus 27.2 months) when comparing patients in the lowest CRBN expression quartile versus those with higher expression [104]. Notably, as CRBN expression decreases in MM patients who develop resistance to Lenalidomide therapy, this does not affect sensitivity to Bortezomib, Melphalan and Dexamethasone [101, 105]. Low levels of the CRBN binding protein IKZF1 and high levels of another CRBN binding protein Karyopherin Subunit Alpha 2 (KPNA2) also correlated with lack of response to Pomalidomide and/or OS [106]. Specifically, patients with low IKZF1 expression had a median OS of 7.3 months compared with 27.2 months in those with higher IKZF1 expression which was also correlated with a similar pattern of PFS (4.9 vs. 7.3 months) [106].

In relapsed/refractory MM patients, the majority (88%) of whom were refractory to an IMiD, an increased prevalence of mutations in the Ras pathway genes KRAS, NRAS and/or BRAF (72%), as well as TP53 (26%), CRBN (12%) and CRBN pathway genes (10%) were observed [107]. Notably, all CRBN-mutated patients and 91% of the CRBN pathway-mutated patients were unresponsive to IMiD based treatment. Moreover, three patients with CRBN mutations at the time of IMiD resistance did not possess these genetic aberrations at the time of IMiD sensitivity. Importantly, the introduction of these mutations in MM cells conferred Lenalidomide resistance *in vitro* [107]. Finally, a pre-clinical study has demonstrated that Lenalidomide resistant MM models over-express the hyaluronan (HA)-binding protein CD44, a downstream Wnt/β-catenin transcriptional target [108]. Consistent with this hypothesis, Lenalidomide resistant MM cell lines show greater adhesion to bone marrow stromal cells. Inhibition of CD44 by application of the humanised monoclonal anti-CD44 antibody RO5429083 induced a modest anti-proliferative effect whilst shRNA-mediated CD44 knockdown resulted in a marked re-sensitisation to Lenalidomide [108].

3.1.2. Clinical studies to circumvent resistance

Whilst the CRBN pathway has been shown to be pivotal in IMiD responsiveness, no clinical studies have eventuated that make use of this important biology as a strategy to overcome resistance to IMiDs and many questions remain such as how much functional CRBN is actually required to maintain IMiD sensitivity. Despite the controversies surrounding CRBN, activating mutations in Ras pathway components, such as KRAS G12D and BRAF V600E, could potentially be targeted with existing compounds in MM patients harbouring these mutations [109]. Such studies have not yet been conducted, although two patients with BRAF V600E positive relapsed/refractory MM achieved significant reductions in tumour burden when treated with the BRAF inhibitor Vemurafenib whilst a patient with highly resistant and rapidly progressive MM also harbouring the BRAF V600E mutation achieved a rapid and sustained response with dual BRAF and MEK inhibition [110].

4. Monoclonal antibodies

Binding of monoclonal antibody (mAb) to its target antigen on MM cells has been shown to induce cell death through several mechanisms including antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), antibody-dependent cellular phagocytosis (ADCP), induction of apoptosis through FcγR-mediated crosslinking of tumour-bound antibodies and modulation of target antigen enzymatic activity after antibody binding [111]. Three mAbs, Daratumumab, Elotuzumab and Pembrolizumab have advanced to phase III clinical trials with Daratumumab the most successful of these.

CD38 is variably expressed on haemopoietic and some non-haemopoietic cells with surface expression depending on the differentiation and activation status of the cell. High cell surface expression occurs on benign and malignant plasma cells [111] with the fully-humanised anti-CD38 mAb Daratumumab demonstrating impressive outcomes when combined with Bortezomib (CASTOR) or Lenalidomide (POLLUX) in the relapsed/refractory MM setting [112, 113]. Other CD38 mAbs, such as Isatuximab (chimeric) and MOR202 (fully human), with differing biological activities from Daratumumab are currently being evaluated in clinical trials (Isatuximab: NCT03275285, NCT03319667, NCT02990338; MOR202: NCT01421186). Elotuzumab binds to signalling lymphocytic activation molecule family member 7 (SLAM7) reducing MM cell binding to bone marrow stroma and activating ADCC [114]. Interestingly, whilst no responses to Elotuzumab as a single agent were observed, the addition of Elotuzumab to Lenalidomide and Dexamethasone in relapsed/refractory MM patients (ELOQUENT-2 trial) resulted in improvements in ORR and PFS, and Elotuzumab is currently the subject of ongoing clinical trials (NCT01891643, NCT02495922, NCT01335399) [115]. Pembrolizumab targets the programmed death 1 (PD-1)/programmed cell death ligand 1 (PD-L1) pathway, a critical initiator of immune activation, playing a role in mediating tolerance [116]. However, two phase III trials KEYNOTE-183 and KEYNOTE-185 have recently been suspended by the US Food and Drug Administration due to more deaths being observed in the Pembrolizumab arms and further information on the use of Pembrolizumab in MM is pending.

4.1. Mechanisms of resistance to monoclonal antibodies

4.1.1. Pre-clinical/clinical findings

The relatively recent addition of mAbs to MM pharmacotherapy means there is a paucity of studies examining resistance mechanisms although these are now being explored with their increasing clinical use (**Table 1** and **Figure 1C**). Examination of CD38 expression on MM cells in 102 patients treated with Daratumumab monotherapy has been insightful [117]. With regard to the effect of Daratumumab on residual bone marrow plasma cells, two important points were clear from this analysis. Firstly, CD38 cell surface expression on plasma cells is highest before Daratumumab treatment and is significantly decreased during treatment. At the time of progressive disease, plasma cells isolated from the bone marrow of these patients exhibited low expression of CD38 suggesting Daratumumab therapy would be less effective, a finding corroborated previously [76]. Secondly, pre-treatment CD38 expression on the surface of MM cells was higher in patients who achieved at least a PR compared to those who did not. Recently, it was shown that Daratumumab-CD38 complexes and accompanying cell membrane are actively transferred from MM cells to monocytes and granulocytes in a process called trogocytosis that was also associated with reduced MM cell surface expression of CD49d, CD56 and CD138 [118]. However, Daratumumab-induced reductions in CD38 expression on MM cells occur in patients with deep and durable responses suggesting reductions in CD38 alone are not responsible for Daratumumab resistance [118]. Cell surface expression of the complement-inhibitory proteins, CD46, CD55 and CD59, was not associated with clinical response but significantly increased only at the time of disease progression. Furthermore, all-*trans* retinoic acid increased CD38 expression whilst decreasing expression of CD55 and CD59 on MM cells from patients who developed Daratumumab resistance to approximately pre-treatment levels, resulting in enhancement of Daratumumab-mediated CDC [117].

In addition to the cell surface expression of target antigens on MM cells, several other potential mechanisms of resistance to mAbs may be at play. Soluble forms of CD38 [119] and SLAM7 [120] may affect the efficacy of Daratumumab and Elotuzumab, respectively. Another potential mechanism of resistance is the development of neutralising antibodies to the therapeutic antibody. This phenomenon was noted in 39% of patients treated with single agent Elotuzumab resulting in more pronounced effects on serum Elotuzumab concentrations [121]. Furthermore, in the ELOQUENT-2 trial, 15% of patients developed anti-Elotuzumab antibodies on at least one occasion [115], however, antibodies directed against Daratumumab have to this day not been detected. Other factors that may contribute to the clinical efficacy of mAb therapy include the frequency and activity of effector immune cells [122], Fcγ receptor polymorphisms [123] and even KIR and HLA genotypes [124].

4.1.2. Clinical studies to circumvent resistance

Whilst the mechanisms of resistance to mAbs are being elucidated, clinical studies specifically designed to overcome these biological processes are largely lacking with the exception of an ongoing phase I/II trial of Daratumumab in combination with all-*trans* retinoic acid for patients with relapsed/refractory MM (NCT02751255).

5. Other factors potentially influencing resistance to myeloma therapies

5.1. Cytogenetics, mutation patterns and clonal evolution

Cytogenetic abnormalities in MM are broadly divided into copy number changes or translocations, most commonly involving the immunoglobulin heavy chain gene [125]. Various cytogenetic abnormalities were shown to be associated with the likelihood of durable responses to therapy but they do not directly explain mechanisms of drug resistance or disease progression [126]. High risk genetic features frequently result in the dysregulation of transcription factors or tumour suppressors and include t(4;14), t(14;16), t(16;20), del(17p) and copy number changes of chromosome 1, which are used for stratifying MM patients in clinical trials and are now becoming important in guiding therapy in routine practice [126]. For example, the EMN02/HO95 study demonstrated the benefit of double autologous stem cell transplantation in patient with high-risk genetics, essentially negating the adverse prognosis of high genetic risk MM [127]. Similarly, the addition of Bortezomib to induction regimens in patients receiving HDM/ASCT may partially overcome cytogenetically defined poor risk [128]. On the other hand, patients with trisomies may respond particularly well to lenalidomide based protocols [129]. Mutational events such as those involving p53 are associated with particularly poor PFS, however, the significant heterogeneity of point mutational events elucidated in whole exome sequencing studies means generalisations of such molecular changes are not possible [130].

The development of whole exome sequencing and copy number profiling was combined with cytogenetics in a landmark paper by a consortium of European and American groups [131]. This elegant paper demonstrated that the majority of MM patients had multiple sub-clones present at the time of diagnosis and that within sub-clones there could be differing mutational events potentially driving behaviour [131]. When serial MM samples were analysed, diverse patterns of clonal evolution were detected. In some cases, simple clonal selection could be observed following a linear pattern of clonal evolution [131]. Differential clonal responses could explain the clinical observation that a MM patient may respond to a treatment initially, lose this response, respond to another treatment and at the time of subsequent relapse respond again to the initial therapy [132]. Branching evolution was also observed in some progressing patients [131]. During disease evolution differing processes may contribute to the mutational repertoire and the relative contributions may vary over time in the same patient resulting in mutational heterogeneity, frequently with very few recurrent genes [131].

5.2. The myeloma stem cell

Identification of the multiple myeloma stem cell (MMSC) has been a challenge predominantly because an agreed phenotype with MM propagating potential has not been definitively established, in part due to differences in experimental techniques and assays. The dominant viewpoint is that clonotypic CD138$^-$ cells represent MMSCs, however, some researchers have also shown that clonotypic CD138$^+$ plasma cells have properties of cancer stem cells such as self-renewal, tumour-initiating potential and drug resistance [133, 134]. Controversy also exists

as to whether the MMSC derives from a clonotypic B cell (CD19$^+$CD138$^-$) or clonotypic non-B cell (CD19$^-$CD138$^{+/-}$). Clonotypic B cells were found to be resistant to a range of anti-MM therapies including Bortezomib and Lenalidomide and possessed a high drug efflux capacity [135]. However, clonotypic non-B cells have also been shown in many studies to result in robust MM reconstitution in the absence of a CD19$^+$ population [136]. To shed some light on this dichotomy with respect to clonotypic non-B cells, there appears to be an interconversion between undifferentiated pre-plasma cells (CD19$^-$CD138$^-$) and differentiated plasma cells (CD19$^-$CD138$^+$) thus representing reversible, bi-directional phenotypic and functional states that share MMSC activity [137]. Furthermore, the pre-plasma cells were found to be more quiescent, primarily located at extramedullary sites, and up to 300-fold more drug resistant to agents including Bortezomib [137]. These informative findings imply phenotypic and functional plasticity between undifferentiated and differentiated clonotypic plasma cells which could explain why differentiated MM plasma cells possess clonogenic capacity and also reconciles inconsistencies surrounding the MMSC phenotype.

Several factors have been attributed to the MMSC that confer drug resistance. (1) Side population (SP) MM cells, which possess stem-like properties, show stronger activity of several ABC transporters when compared to main population (MP) cells [138]. (2) High levels of aldehyde dehydrogenase (ALDH) have been demonstrated in CD138$^-$ plasma cells compared to their CD138$^+$ counterparts rendering the CD138$^-$ population more resistant to certain chemotherapeutic agents which result in the generation of toxic aldehyde intermediates that are metabolised by ALDH1 [135]. In one study, forced expression of member A1 of the ALDH1 family of proteins resulted in resistance to Bortezomib [139]. (3) Increased expression of Bcl-2 family members in MMSCs expressing the retinoid acid receptor alpha 2 (RARα2) endowed these cells with increased drug resistance [140], and more recently, increased expression of Bruton's tyrosine kinase (BTK) in MMSCs also induced drug resistance [141]. (4) CD19$^-$CD138$^+$ plasma cells and CD19$^-$CD138$^-$ pre-plasma cells harbour MMSC activity but exhibit differential resistance to treatment since pre-plasma cells are more quiescent than plasma cells, shown by a lower proportion of these cells in S phase of the cell cycle [137]. Finally, (5) the Wingless (Wnt), Hedgehog and Notch signalling pathways are all highly active in MMSCs and may be responsible for maintaining stem cell properties, propagating MM and promoting therapeutic resistance together with a supportive and protective BMME [142].

6. Conclusion

Continued improvements in the efficacy and toxicity profiles of an ever-expanding number of novel MM therapies are challenging the current paradigm of high-dose therapy and autologous stem cell transplantation for newly-diagnosed MM. However, despite these advances, resistance to novel agents has been observed and will continue to be observed, requiring innovative ways to circumvent this problem. Changing therapy from one novel agent containing treatment regimen to a different one upon MM progression or relapse is reasonable, however, there is often little scientific basis for choosing the sequence of such regimens and the era of precision medicine for MM patients remains distant. Moreover, the inability to tailor treatment

regimens for an individual patient based on the biology of their MM due to government mandated prescribing restrictions likely contributes to inadequate responses and drug resistance.

In addition to those discussed, there are other potential mechanisms through which resistance to novel therapies in MM may occur, such as the role miRNAs play in promoting MM, and this list is likely to increase. However, despite the varied resistance mechanisms reported to date, the survival of patients with MM continues to improve. Whilst genetic profiling has established a so-called high-risk group of MM patients, these genetic changes do not specifically explain why resistance to a particular novel agent develops. Thus, in this Chapter, an exposition of specific biological aberrations that have been linked to drug resistance has been presented.

Acknowledgements

Conflict of interest

The authors have no conflicts of interest to declare.

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