Treatment optimization for multiple myeloma: schedule-dependent synergistic cytotoxicity of pomalidomide and carfilzomib in *in vitro* and *ex vivo* models

Despite recent advances in the treatment of multiple myeloma (MM), the disease continues to remain incurable for the vast majority of patients. To improve their outcomes, combinations of different classes of drugs with distinct modes of action are currently under evaluation. The rationale supporting the continuous development of alternative treatment strategies and validation of new drug targets is based on the recent understanding that clonal evolution and the bone marrow microenvironment contribute to the acquisition of drug resistance and disease progression.[1-4] Hence, the choice of the most effective therapies and optimal drug sequencing for MM should take into consideration the different biological features of the disease. While the efficacy of proteasome inhibitors and immunomodulatory drugs in combination with dexamethasone or combined with each other plus dexamethasone has long been demonstrated, their synergistic cytotoxicity may be further exploited by using opti-

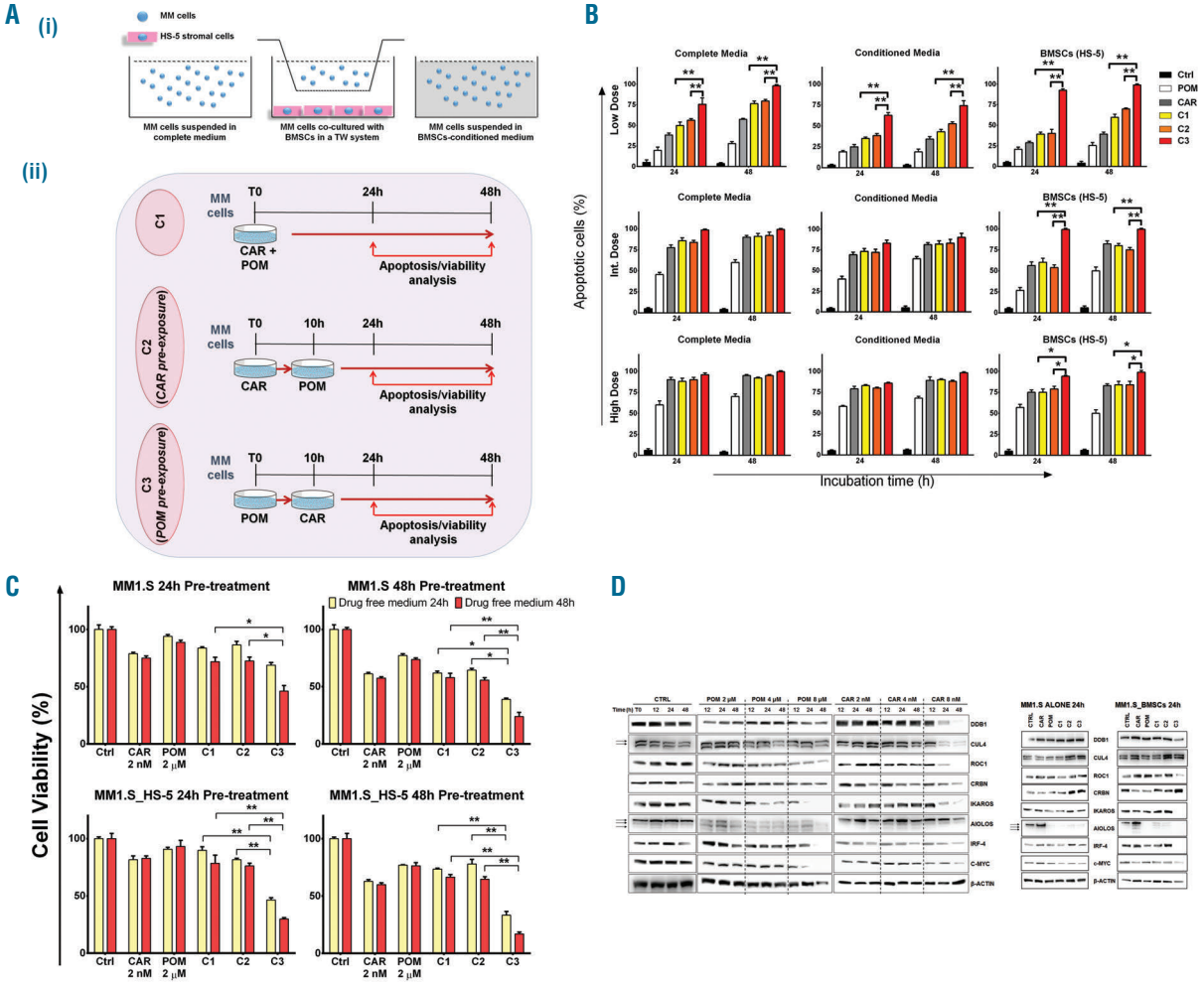

Figure 1. Study design and apoptosis analysis in the MM1.S cell line treated in different experimental conditions with or without bone marrow stromal cells. (A) Diagram of the culture system used in this study (i): MM cells cultured in complete medium, or MM cells in a transwell (TW) culture system with bone marrow stromal cells (BMSCs; i.e. the HS-5 cell line) (enabling soluble factor movement and cell interactions without direct cell-cell contact), or MM cells suspended in medium conditioned in the prior presence of BMSCs (i.e. the HS-5 cell line). (ii) Experimental design showing the C1 scheme, in which cells were simultaneously exposed to both carfilzomib (CAR) and pomalidomide (POM) for up to 48 h, and the C2 and C3 sequential combination schemes, in which cells were first incubated with either CAR or POM for the first 10 h (C2 and C3 schemes, respectively) and then exposed to either POM or CAR for up to 48 h (C2 and C3 schemes, respectively). Apoptosis was determined 24 h and 48 h after first drug exposure. (B) Apoptosis rate of MM1.S cell line cultured in three different experimental conditions. Flow cytometric determination of early and late apoptosis of MM cells upon exposure to either CAR or POM used either as single agents in a sequential scheme or in combination (as described above) for up to 48 h. Staining for annexin V and propidium iodide showed an increased fraction of double-positive events after 24 h of incubation for all conditions tested. Three different drug concentrations were used for each agent: a low dose (CAR 2 nM and POM 2 μM), an intermediate dose (CAR 4 nM and POM 4 μM) and a high dose (CAR 8 nM and POM 8 μM). MM1.S cells were cultured in complete medium, in a conditioned medium derived from HS-5 stromal cells, and co-cultured with HS-5 cells. Values shown in histograms are the means ± standard deviations of two independent experiments. *P<0.05 and **P<0.01. (C) Cell death commitment assay of MM1.S cells was performed to evaluate the irreversible impact of the different combination schedules on cell viability. MM1.S cells were exposed to either CAR (2 nM) or POM (2 μM) used either as a single agent or in combination (i.e. the C1 scheme) or in a sequential scheme (i.e. the C2 and C3 schemes) for up to 48 h followed by drug washout and further incubation in drug-free medium for another 48 h. Values shown in the histograms are the means ± standard deviations of two independent experiments. *P<0.05 and **P<0.01. (D) Western blotting analysis of IKZF's pathway. Time course analysis of IKAROS's pathway in MM1.S cells treated with different concentrations of CAR and POM for up to 48 h. MM1.S cells alone or co-cultured with BMSCs (using a TW system) were treated as indicated for 24 h and then an immunoblotting assay was performed to assess the impact of different schedules on IKZF's pathway.

mized schedules. Preclinical studies suggested that the timing and dosing schedules of immunomodulatory drugs given in combination with proteasome inhibitors are critical, indicating that established treatment regimens need to be carefully re-evaluated to maximize their anti-tumor activities.[5]

In this study, we investigated the cytotoxic interactions between proteasome inhibitors (carfilzomib or bortezomib) and immunomodulatory drugs (pomalidomide or lenalidomide) by determining the optimal schedule of administration of the two classes of drugs both in *in vitro* models, including bone marrow microenvironment simulation, and *ex vivo* using patient-derived samples. For this purpose, we explored three different combination schedules: simultaneous exposure to both immunomodulatory drugs and proteasome inhibitors (C1 scheme) and sequential exposure to drug combinations in which cells were initially treated with proteasome inhibitors for 10 h followed by immunomodulatory drugs (C2 scheme) or with immunomodulatory drugs for 10 h followed by proteasome inhibitors (C3 scheme) (Figure 1A). For each drug, three different concentrations of proteasome inhibitors and immunomodulatory drugs were used: *low dose* (2 nM and 2 μM, respectively), *intermediate dose* (4 nM and 4 μM, respectively) and *high dose* (8 nM and 8 μM, respectively).

Overall, we found that the administration of immunomodulatory drugs prior to proteasome inhibitors was associated with the greatest response, in terms of cell killing, under any conditions tested as early as 24 h. Indeed, by comparing Annexin V/PI positive cells cells between samples, we found that MM1.S cells incubated with pomalidomide for 10 h prior to exposure to carfilzomib (i.e. the C3 scheme) had a higher rate of apoptosis in comparison with the rates in cells treated with the C1 and C2 schemes (Figure 1B; for other cell lines, see *Online Supplementary Material*). We next exposed MM cells to both drugs in the presence of conditioned medium prepared from bone marrow stromal cells (i.e. HS-5 cells), or of bone marrow stromal cells using a transwell system (enabling soluble factor movement and cell interactions without direct cell-cell contact) to mimic bone marrow cross-signaling.[6-8] Overall, our data suggested that the marked anti-tumor activity of the administration of pomalidomide prior to carfilzomib was retained even in the presence of bone marrow stromal cell stimuli. Non-direct contact of MM cells with bone marrow stromal cells decreased MM cell death induced by carfilzomib and pomalidomide as single agents. The protective effect of bone marrow stromal cells was still present in cells incubated simultaneously with carfilzomib and pomalidomide, and in cells treated with the carfilzomib pre-

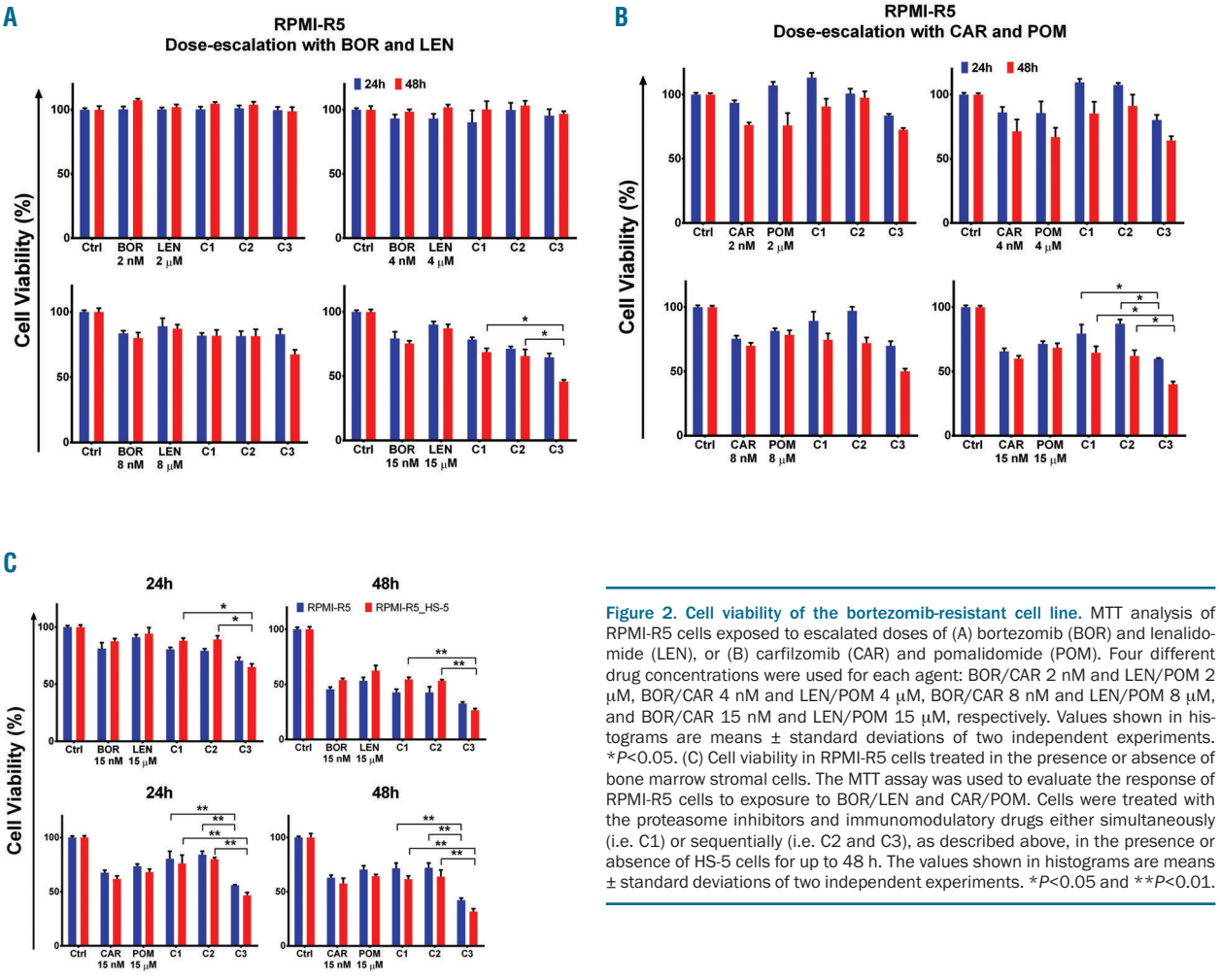

Figure 2. Cell viability of the bortezomib-resistant cell line. MTT analysis of RPMI-R5 cells exposed to escalated doses of (A) bortezomib (BOR) and lenalidomide (LEN), or (B) carfilzomib (CAR) and pomalidomide (POM). Four different drug concentrations were used for each agent: BOR/CAR 2 nM and LEN/POM 2 μM, BOR/CAR 4 nM and LEN/POM 4 μM, BOR/CAR 8 nM and LEN/POM 8 μM, and BOR/CAR 15 nM and LEN/POM 15 μM, respectively. Values shown in histograms are means ± standard deviations of two independent experiments. *P<0.05. (C) Cell viability in RPMI-R5 cells treated in the presence or absence of bone marrow stromal cells. The MTT assay was used to evaluate the response of RPMI-R5 cells to exposure to BOR/LEN and CAR/POM. Cells were treated with the proteasome inhibitors and immunomodulatory drugs either simultaneously (i.e. C1) or sequentially (i.e. C2 and C3), as described above, in the presence or absence of HS-5 cells for up to 48 h. The values shown in histograms are means ± standard deviations of two independent experiments. *P<0.05 and **P<0.01.

exposure scheme (Figure 1B). Conversely, in cells treated with pomalidomide prior to carfilzomib, the protective effect of the bone marrow microenvironment was lost, indicating that the pomalidomide pre-exposure schedule was superior to the C1 and C2 schemes.

Subsequently, MM1.S cells were exposed to carfilzomib and pomalidomide as single agents or in different combination schedules for up to 48 h, followed by drug washout, and then grown in drug-free medium for a further 24 h with or without bone marrow stromal cells[9] (Figure 1C). In drug-free medium, cell proliferation was irreversibly affected by short-term (i.e. 24 h) exposure to pomalidomide, and the protective effect of bone marrow stromal cells was lost, with a maximum apoptosis rate observed in the 48 h pre-treatment experiments. Taken together, these data demonstrate that C3 combination therapy was able to overcome the proliferative effect induced by bone marrow stromal cells. It might be hypothesized that pre-incubation with an immunomodulatory drug prevents the interaction between MM cells and the bone marrow microenvironment and primes MM cells to undergo robust cell death in response to subsequent treatment with even low concentrations of proteasome inhibitors. To verify this hypothesis, we performed an immunoblotting assay to determine the molecular events leading to cell death. The mechanism by which immunomodulatory drugs cause the death of MM cells is being gradually elucidated, and the E3 ubiquitin ligase, cereblon (CRBN), has been shown to be the primary target of these drugs.[10] In order to verify the modulation of CRBN's pathway, we first treated MM1.S cells with increasing doses of both carfilzomib and poma-

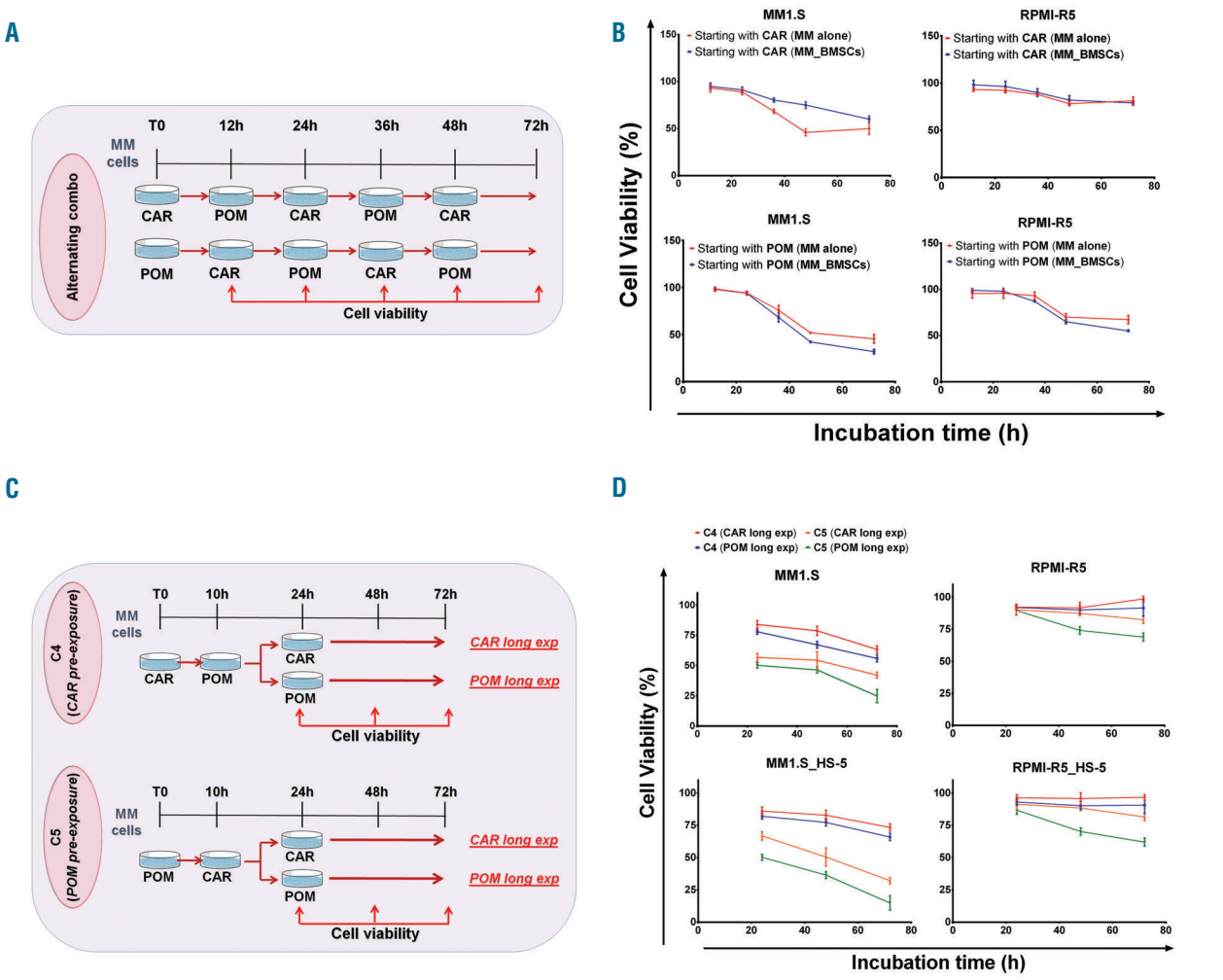

Figure 3. Cell viability of the sensitive MM1.S cell line and resistant RPMI-R5 cell line using "alternating" and "long exposure" drug combination schemes. (A) Experimental design. In the "alternating combo" scheme, MM cells were alternately exposed to carfilzomib (CAR) and pomalidomide (POM) (or POM and CAR) for 12 h each, up to 72 h. Cell viability was analyzed every 12 h up to 72 h after administration of the first drug. (B) Cell viability in the MM1.S and RPMI.R5 cell lines. The MTT assay was used to determine the response of MM1.S and RPMI-R5 cells to alternating exposure to CAR and POM in the presence or absence of bone marrow stromal cells (i.e. HS-5). Values shown in the graphs are means ± standard deviations of two independent experiments. (C) Experimental design of the "long-term exposure" schemes. After pre-exposure to either CAR/POM or POM/CAR according to the C2 and C3 schemes, respectively, MM cells were then treated with prolonged exposure to either CAR for up to 72 h (i.e. C4 CAR-long and C5 CAR-long schemes, respectively) or POM for up to 72 h (i.e. C4 POM-long and C5 POM-long schemes, respectively). Cell viability was analyzed up to 72 h after administration of the first drug. (D) Cell viability in MM1.S and RPMI.R5 cell lines. The MTT assay was used to determine the response of MM1.S and RPMI-R5 cells to both CAR and POM "long exposure" in the presence or absence of bone marrow stromal cells (i.e. HS-5). Values shown in the graphs are means ± standard deviations of two independent experiments.

lidomide for up to 48 h (Figure 1D). We observed time- and dose-dependent degradation of CRBN substrates and downregulation of downstream molecular targets in both carfilzomib- and pomalidomide-treated cells. We next evaluated whether different combination schedules could affect CRBN's pathway, eventually leading to reductions in c-MYC and IRF-4 protein levels in a system mimicking the bone marrow. A marked downregulation of CRBN's pathway was observed in C3-exposed samples as early as 24 h after treatment, whereas partial degradation of IKZF1/3, IRF-4 and c-MYC was detected by performing experiments with the C1 and C2 schemes. Notably, the robust downregulation of the IRF-4/c-MYC axis was directly correlated with the higher apoptotic rate observed in the cells treated with the C3 scheme. Overall, these results reveal that the ensuing downregulation of the c-MYC/IRF-4 axis was a critical factor in the commitment to cell death triggered by the pomalidomide pre-exposure schedule, suggesting that once that pathway is compromised, MM cells can no longer be rescued from growth inhibition (Figure 1D).

We were able to reproduce these results in the bortezomib-resistant cell line RPMI-R5. We first treated MM cells with escalating doses of either bortezomib/lenalidomide or carfilzomib/pomalidomide as shown in Figures 2A and 2B, respectively. Based on cell viability results, we next incubated the RPMI-R5 cell line with bortezomib/carfilzomib and lenalidomide/pomalidomide at the highest doses of 15 nM and 15 μM, respectively, with or without bone marrow stromal cells, for up to 48 h. As illustrated in Figure 2C, we found schedule-dependent synergistic cytotoxicity for the combinations of bortezomib/ lenalidomide and carfilzomib/pomalidomide, although the maximal apoptosis rate was observed in cells pre-exposed to immunomodulatory drugs. The superiority of this schedule was maintained in the bone marrow microenvironment system, strengthening the concept that an optimized schedule including pre-treatment with an immunomodulatory drug might improve the depth and duration of responses.

Moreover, flow cytometry analysis of $CD138^+$ bone marrow plasma cells obtained from six newly diagnosed MM patients confirmed the advantage of treating cells with immunomodulatory drugs prior to proteasome inhibitors (*Online Supplementary Material*).

Finally, to support the conclusion that pre-exposure to immunomodulatory drugs enhances proteasome inhibitor-induced MM cell death, we treated both MM1.S and RPMI-R5 cells with pomalidomide alternated every 12 h with carfilzomib (and *vice versa*) for up to 72 h, with or without bone marrow stromal cells (Figure 3A). As shown in Figure 3B, MM cells were driven to more pronounced cell death when initially exposed to pomalidomide, especially in the presence of the bone marrow microenvironment. Subsequently, we evaluated the impact of pre-exposure to carfilzomib/pomalidomide or pomalidomide/carfilzomib according to the C2 and C3 schemes, respectively, followed by prolonged exposure to carfilzomib for up to 72 h (Figure 3C: C4 carfilzomib-long and C5 carfilzomib-long, respectively) or to pomalidomide for up to 72 h (Figure 3C: C4 pomalidomide-long and C5 pomalidomide-long, respectively). Although, a reduction of cellular viability was observed following both the C4 and C5 schemes in any of tested conditions, the effect was greatest when MM1.S cells were pre-incubated with pomalidomide and subsequently exposed to carfilzomib or pomalidomide for 72 h. This effect was even more evident in the presence of bone marrow stromal cells (Figure 3D). Notably, the C5 pomalidomide-long scheme in the presence of bone marrow stromal cells was the only combination able to overcome the intrinsic drug resistance of RPMI-R5 cells, a finding that again underscored the usefulness of a scheme including pre-treatment with an immunomodulatory drug to maximize the anti-tumor effect.

Although we did not analyze the mechanisms underlying this phenomenon, we speculate that immunomodulatory drugs may interfere with stroma-derived anti-apoptotic signals to MM cells and increase their sensitivity to subsequent treatment with proteasome inhibitors.

The rapid emergence of novel anti-MM agents has raised questions about their optimal combination and/or sequencing. First- and second-generation proteasome inhibitors and immunomodulatory drugs have synergistic mechanisms of action, a fact which has represented the basis for their combined use to form the backbone of many treatments for both newly diagnosed and relapsed/refractory MM. In the present study, we designed several *in vivo* and *ex vivo* models to explore alternative treatment schedules of carfilzomib/pomalidomide and bortezomib/lenalidomide. Interestingly, pre-incubation with an immunomodulatory drug followed by sequential or alternating combinations had the most pronounced anti-myeloma effect. This finding might have a potential impact on the design of future treatment regimens, although the effect was seen predominantly at suboptimal doses. The mechanisms underlying these observations are still unclear but could be explained by competing synergistic and antagonistic effects of both classes of drugs. Further analyses addressing the optimal duration of pre-exposure with immunomodulatory drugs are necessary. Moreover, effects in humans may be different from those in the MM cell models used in this study. Further *in vivo* and preclinical data are needed to confirm the re-definition of the optimal sequencing of these drugs in everyday clinical practice.

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