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Treatment optimization for Multiple Myeloma: schedule-dependent synergistic cytotoxicity of pomalidomide and carfilzomib in an *in vitro* and *ex-vivo* model

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Despite recent advances in the treatment for 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 upon the recent understanding that clonal evolution and bone marrow microenvironment contribute to acquisition of drug resistance and disease progression [1-4]. Hence, the choice of the most effective therapies and optimal drug sequencing for MM should deal with different biologic features of the disease. Whilst the efficacy of proteasome inhibitors (PIs) and immunomodulatory drugs (IMiDs) in combination with dexamethasone or combined with each other plus dexamethasone has long been demonstrated, their synergistic cytotoxicity may be further exploited by using optimized schedules. Preclinical studies suggested that the timing and dosing schedules of IMiDs given in combination with PIs are critical, advising that established treatment regimens need to be carefully re-evaluated to maximize their anti-tumour activities [5].

In this study, we investigated the cytotoxic interactions between PIs (Carfilzomib, CAR, or Bortezomib, BOR) and IMiDs (Pomalidomide, POM, or Lenalidomide, LEN) by determining the optimal schedule of PIs and IMiDs administration both in *in vitro* models, including BM microenvironment simulation, and in *ex-vivo* using patient-derived samples. For this purpose, we explored three different combination schedules which included either the simultaneous exposure to both IMiDs and PIs (i.e. C1 scheme) or exposure to sequential drug combinations (combo) in which cells were initially treated with PIs for 10h followed by IMiDs (i.e. C2 scheme) or with IMiDs for 10h followed by PIs (i.e. C3 scheme) (Figure 1A). For each drug, three different concentrations were used: *low dose* (2 nM and 2 μM for PIs and IMiDs, respectively), *intermediate dose* (4 nM and 4 μM for PIs and IMiDs, respectively) and *high dose* (8 nM and 8 μM for PIs and IMiDs, respectively).

Overall, we found that the administration of IMiDs prior to PIs was associated with the greatest response in terms of cell killing under any conditions tested as early as 24h. Indeed, by comparing Annexin V/PI positive cells between samples, we demonstrated that MM1.S cells incubated with POM 10h prior to CAR exposure (i.e. C3 scheme) had a higher rate of apoptosis in comparison with that seen with 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 (BMSCs) (i.e. HS-5 cells), or of BMSCs using a transwell system (TW) (soluble factors and cells interaction without direct cell-cell contact) to mimic a BM cross signaling [6-8]. Overall, our data suggested that with the administration of POM prior to CAR high anti-tumor activity was retained even in the presence of BMSCs stimuli. Non-direct contact of

MM cells with BMSCs decreased MM cell death induced by CAR and POM as single agents. The protective effect of SCs was still present in cells incubated simultaneously with CAR and POM, and in CAR pre-exposure scheme (Figure 1B). Conversely, in cells treated with POM prior to CAR, the protective effect of BM microenvironment was lost, indicating that POM pre-exposure schedule was superior to C1 and C2 schemes.

Subsequently, MM1.S cells were exposed to CAR and POM as single agents or in different combination schedules for up to 48h, followed by drug washout, and then grown in drug-free medium for additional 24h with or without BMSCs [9] (Figure 1C). In drug-free medium, cell proliferation was irreversibly affected by short-term (i.e. 24h) exposure to POM, and the protective effect of BMSCs was lost, with a maximum apoptosis rate observed in 48h pre-treatment experiments. Taken together, these data demonstrate that C3 combination therapy was able to overcome the proliferative effect induced by BMSCs. It might be hypothesized that pre-incubation with an IMiD prevents the interaction between MM cells and the BM microenvironment and primes MM cells to undergo robust cell death in response to subsequent treatment with even low concentrations of PIs. To verify this hypothesis, we performed an immunoblotting assay to determine the molecular events leading to cell death. The mechanism by which IMiDs cause the death of MM cells has been gradually uncovered, and the E3 ubiquitin ligase, cereblon (CRBN), was shown to be the primary target of IMiDs [10]. In order to verify the modulation of CRBN's pathway, we first treated MM1.S cells with increasing doses of both CAR and POM for up to 48h (Figure 1D). We observed a time and dose dependent degradation of CRBN substrates and downregulation of downstream molecular targets in both CAR- and POM-treated cells. We next evaluated whether different combination schedules affect CRBN's pathway, eventually leading to reductions in c-MYC and IRF-4 protein levels in a BM mimicking system. A marked downregulation of CRBN's pathway was observed in C3-exposed samples as early as 24h, whereas a partial degradation of IKZF1/3, IRF-4 and c-MYC was detected by performing experiments with C1 and C2 schemes. Notably, this robust downregulation of IRF-4/c-MYC axis was directly correlated with the higher apoptotic rate observed in 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 in POM pre-exposure schedule, suggesting that once that pathway is compromised, MM cells can no longer be rescued from growth inhibition (Figure 1D).

Furthermore, we were able to reproduce these results in the BOR-resistant cell line RPMI-R5. We first treated MM cells with a dose-escalation of either BOR/LEN or CAR/POM as shown in Figures 2A and 2B, respectively. Based on cell viability results, we next incubated the R5 cell line with BOR/CAR and LEN/POM at the highest doses of 15 nM and 15 μM, respectively, with or without

BMSCs for up to 48h. As summarized in Figure 2C, we showed a schedule-dependent synergistic cytotoxicity for the combination of BOR/LEN and CAR/POM, although the maximal apoptosis rate was observed in cells pre-exposed to IMiDs. The superiority of this schedule was kept up in the BM microenvironment system, strengthening the concept that an optimized schedule including pre-treatment with an IMiD might improve the depth and duration of response.

Moreover, flow cytometry analysis of $CD138^+$ BM plasma cells obtained from six newly diagnosed MM patients, confirmed again the advantage of treating cells with IMiDs prior to PIs (*Online supplementary material*).

Finally, to support the conclusion that pre-exposure to IMiDs enhanced PI-induced MM cell death, we treated both MM1.S and RPMI-R5 cells with POM alternated every 12h to CAR (and *vice versa*) for up to 72h, with or without BMSCs (Figure 3A). As shown in Figure 3B, MM cells were pushed to undergo a more pronounced cells death when initially exposed to POM, especially in the presence of BM microenvironment. Subsequently, we evaluated the impact of pre-exposure to CAR/POM or POM/CAR according to C2 and C3 schemes, respectively, followed by prolonged exposure to CAR for up to 72h (Figure 3C: C4 CAR long and C5 CAR long, respectively) or to POM for up to 72h (Figure 3C: C4 POM long and C5 POM long, respectively). Although, a reduction of cellular viability in both C4 and C5 schemes was observed in any of tested conditions, the greatest effect was demonstrated when MM1.S cells were pre-incubated with POM and subsequently exposed to CAR or POM for 72h. This effect was even more evident in the presence of BMSCs (Figure 3D).

Notably, C5 POM long scheme in the presence of BMSCs 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 IMiD to maximize the anti-tumor effect.

Although we did not analyze the mechanisms behind this phenomenon, we might speculate that IMiDs may interfere with stroma-derived anti-apoptotic signals to MM cells and increase their sensitivity to subsequent treatment with PIs.

The rapid emergence of novel anti-MM agents has raised the question about their optimal combination and/or sequencing. First and second generation PIs and IMiDs have synergistic mechanisms of action which have 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 CAR/POM and BOR/LEN. Interestingly, pre-incubation with IMIDs followed by sequential or alternating combos had the most pronounced anti-myeloma effect. These findings 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 and may be explained by competing synergistic and antagonistic effects of both classes of drugs. Further analyses addressing the optimal duration of pre-exposure with IMiDs will be planned in future studies. Moreover, we are aware that the human setting might be different from 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 the everyday clinical practice.

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Authorship

Contribution: E.B. and C.T. designed research, analyzed and interpreted the data and wrote the manuscript. E.B. performed most experiments and statistical analyses. E.Z., P.T., L.P., K.M. and S.R. collected primary samples. M.M. and B.S. performed samples processing and progenitor cells isolation. All co-authors contributed to design the experiments and collect data. M.C. critically revised the paper, which was approved by the other Authors.

Disclosure. The authors have declared no conflicts of interest.

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FIGURE LEGENDS

Figure 1. Study design and apoptosis analysis in MM1.S cell line treated in different experimental conditions with or without BMSCs. (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 BMSCs (i.e. HS-5 cells line) (soluble factors and cells interaction without direct cell-cell contact), or MM cells suspended in medium conditioned in the prior presence of BMSCs (i.e. HS-5 cells line). **(ii)** Experimental plan. C1 scheme, in which cells were simultaneously exposed to both CAR and POM for up to 48h; C2 and C3 "sequential combo" schemes, in which cells were firstly incubated with either CAR or POM for the first 10h (C2 and C3 schemes, respectively) and subsequently exposed to either POM or CAR for up to 48h (C2 and C3 schemes, respectively. Apoptosis analysis was performed after 24h and 48h from first drug exposure. **(B)** Apoptosis rate of MM1.S cell line cultured in three different experimental conditions. Early and late apoptotic events 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 48h were measured by flow cytometry. Staining for Annexin V and PI showed an increased fraction of Annexin V^+PI^+ events after 24h of incubation for all conditions tested. Three different drug concentrations were used for each agent: *low dose* (CAR 2nM and POM 2 μM, respectively), *intermediate dose* (CAR 4 nM and POM 4 μM, respectively) and *high dose* (CAR 8 nM and POM 8 μM, respectively). 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 mean ± SD of two independent experiments. * $p<0.05$ and ** $p<0.01$. Control-*black bars*, Pomalidomide-*white bars*, Carfilzomib-*grey bars*, C1-*yellow bars*, C2-*orange bars*, C3-*red bars*. **(C)** Cell death commitment assay of MM1.S cells was performed to evaluate the irreversible impact on cell viability of the different combination schedules. 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. C1 scheme) or in a sequential scheme (i.e. C2 and C3 schemes) for up

to 48h followed by drug washout and further incubation in drug free medium for additional 48h. Values shown in histograms are mean ± SD of two independent experiments. * $p<0.05$ and ** $p<0.01$. 24h in drug free medium-*blue bars*, 48h in drug free medium-*red bars*. **(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 48h. MM1.S cells alone or co-coltured with BMSCs (using a TW system) were treated as indicated for 24h and then an immunoblotting assay was performed to assess the impact of different schedule on IKZF's pathway.

Figure 2. Cell viability in BOR resistant cell line. MTT analysis of RPMI-R5 cells exposed to escalated doses of BOR and LEN **(A),** or CAR and POM **(B).** Four different drug concentrations were used for each agent: BOR/CAR 2nM 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 mean ± SD of two independent experiments. * $p<0.05$. **(C)** Cell viability in RPMI-R5 cells treated in the presence or absence of BMSCs. MTT assay response of RPMI-R5 cells to both BOR/LEN and CAR/POM exposure. Cells were treated with PIs and IMiDs 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 48h. Values shown in histograms are mean ± SD of two independent experiments. * $p<0.05$ and ** $p<0.01$. MM cells alone-*blue bars*, MM cells co-coltured with HS-5-*red bars*.

Figure 3. Cell viability in the sensitive MM1.S cell line and resistant RPMI-R5 cell line using an "alternating" and "long exposure" drug combo schemes. (A) Experimental plan: "alternating combo" scheme. MM cells were alternatively exposed to CAR and POM (or POM and CAR) for 12 h each, up to 72h. Cell viability analysis was performed every 12h and up to 72h after

administration of the first drug. **(B)** Cell viability in MM1.S and RPMI.R5 cell lines. MTT assay response of MM1.S and RPMI-R5 cells alternatively exposed to CAR and POM in the presence or absence of BMSCs (i.e. HS-5). Values shown in graphs are mean ± SD of two independent experiments. **(C)** Experimental plan: "long-term exposure" schemes. After pre-exposure to either CAR/POM or POM/CAR according to C2 and C3 schemes, respectively, MM cells were then treated with long-term exposure to either CAR for up to 72h (i.e. C4 CAR long and C5 CAR long schemes, respectively) or POM for up to 72h (i.e. C4 POM long and C5 POM long schemes, respectively). Cell viability analysis was performed up to 72h after administration of the first drug. **(D)** Cell viability in MM1.S and RPMI.R5 cell lines. MTT assay response of MM1.S and RPMI-R5 cells to both CAR and POM "long exposure" in the presence or absence of BMSCs (i.e. HS-5). Values shown in graphs are mean ± SD of two independent experiments.

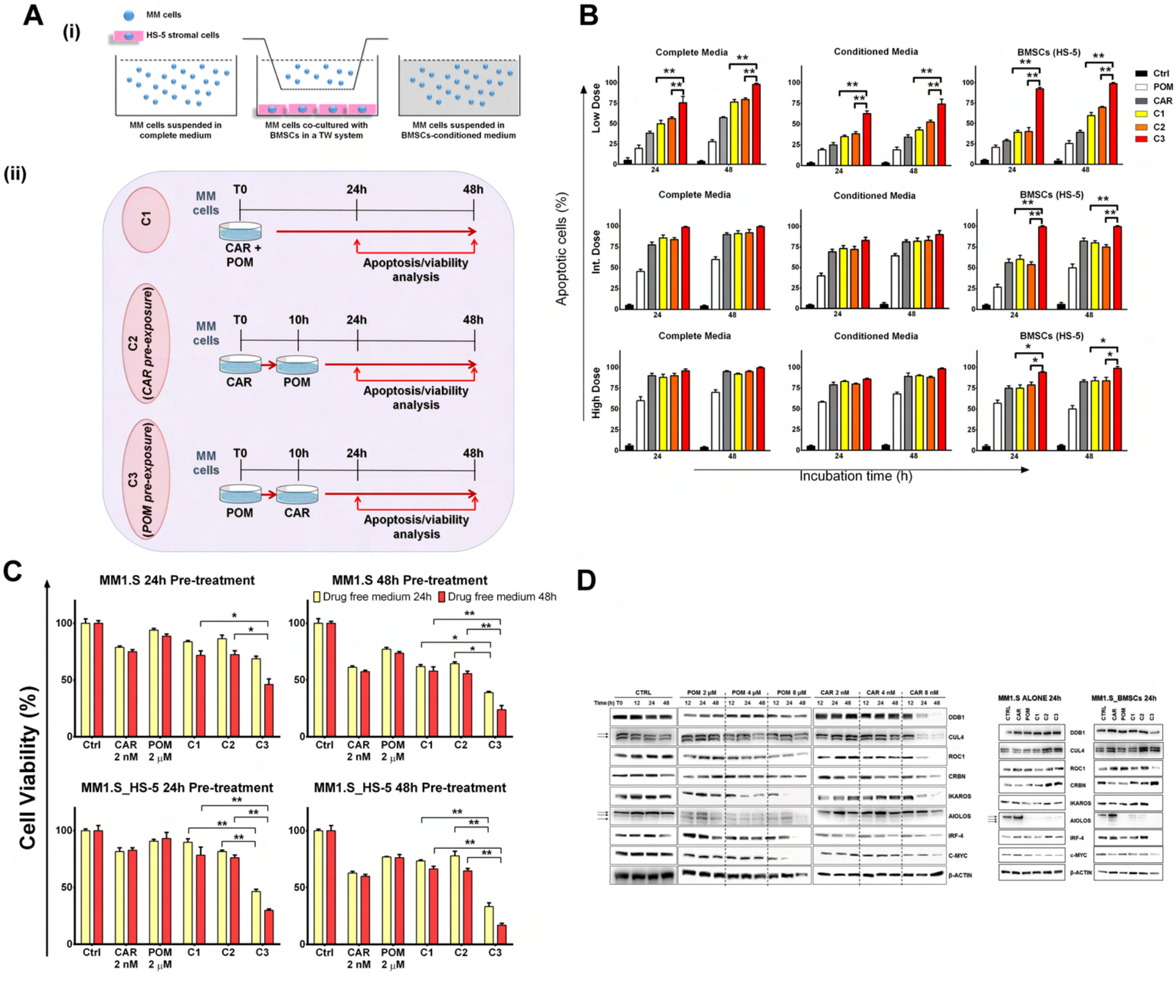

A
(i)
MM cells
HS-5 stromal cells
MM cells suspended in complete medium
MM cells co-cultured with BMSCs in a TW system
MM cells suspended in BMSCs-conditioned medium
(ii)
C1
MM cells
T0
24h
48h
CAR + POM
Apoptosis/viability analysis
C2 (CAR pre-exposure)
10h
CAR
POM
C3 (POM pre-exposure)
B
Complete Media
Conditioned Media
BMSCs (HS-5)
Low Dose
Int. Dose
High Dose
Apoptotic cells (%)
Incubation time (h)
Ctrl
POM
CAR
C1
C2
C3
C
MM1.S 24h Pre-treatment
MM1.S 48h Pre-treatment
Drug free medium 24h
Drug free medium 48h
MM1.S_HS-5 24h Pre-treatment
MM1.S_HS-5 48h Pre-treatment
Cell Viability (%)
Ctrl
CAR 2 nM
POM 2 μM
C1
C2
C3
D
CTRL
POM 2 μM
POM 4 μM
POM 8 μM
CAR 2 nM
CAR 4 nM
CAR 8 nM
Time (h)
DDB1
CUL4
ROC1
CRBN
IKAROS
AIOLOS
IRF-4
C-MYC
β-ACTIN
MM1.S ALONE 24h
MM1.S_BMSCs 24h

Figure 1

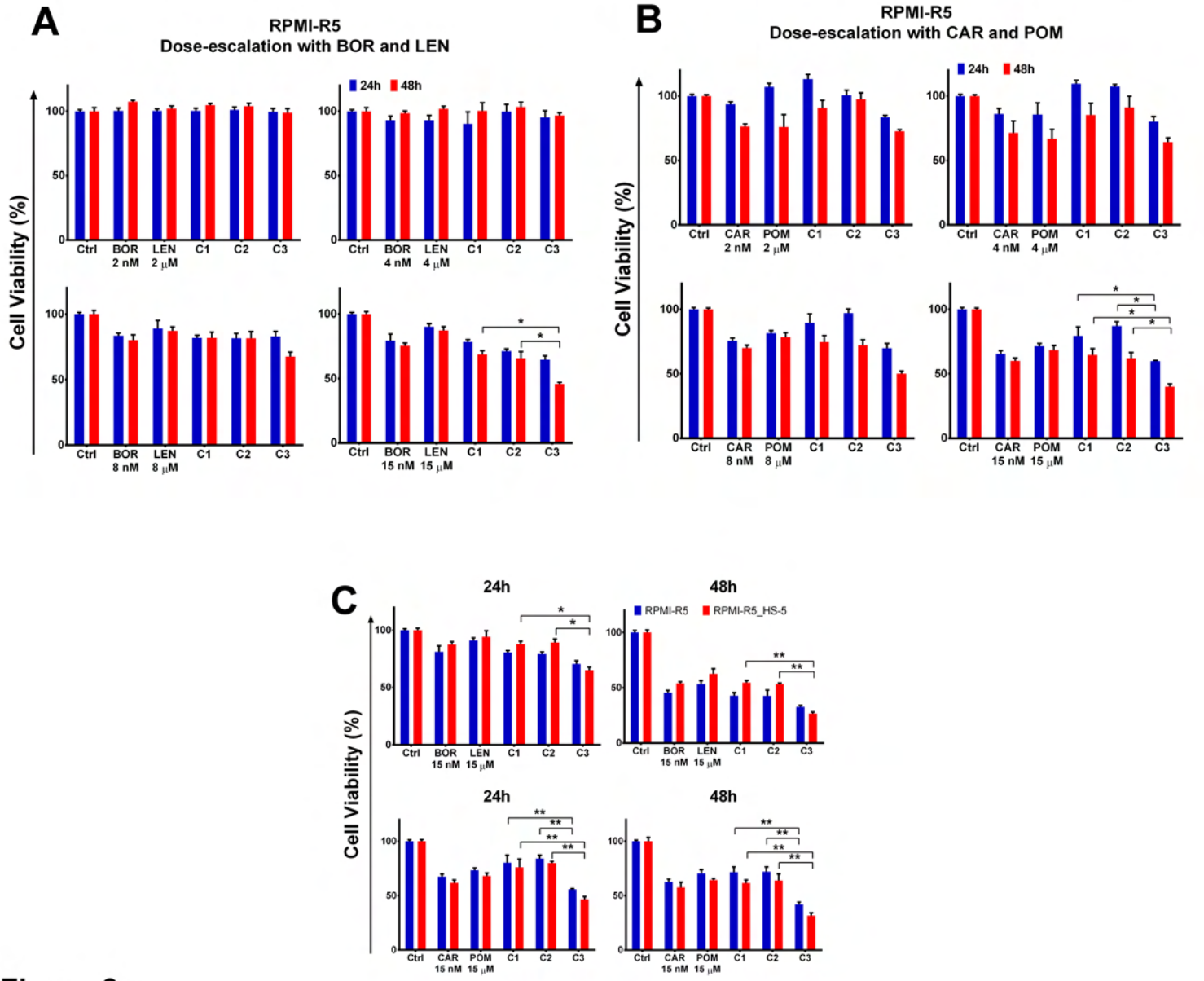
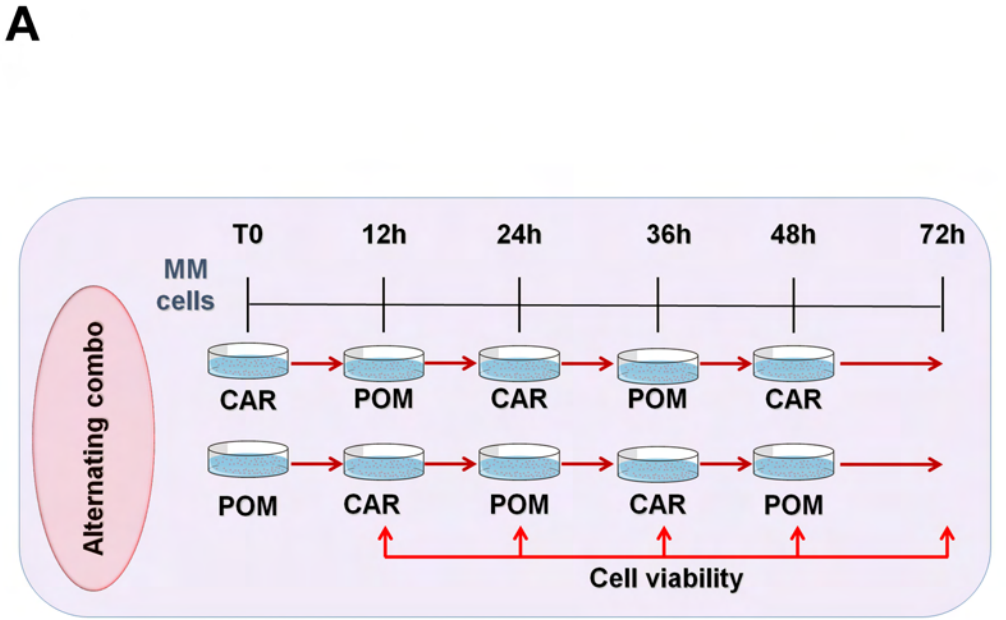
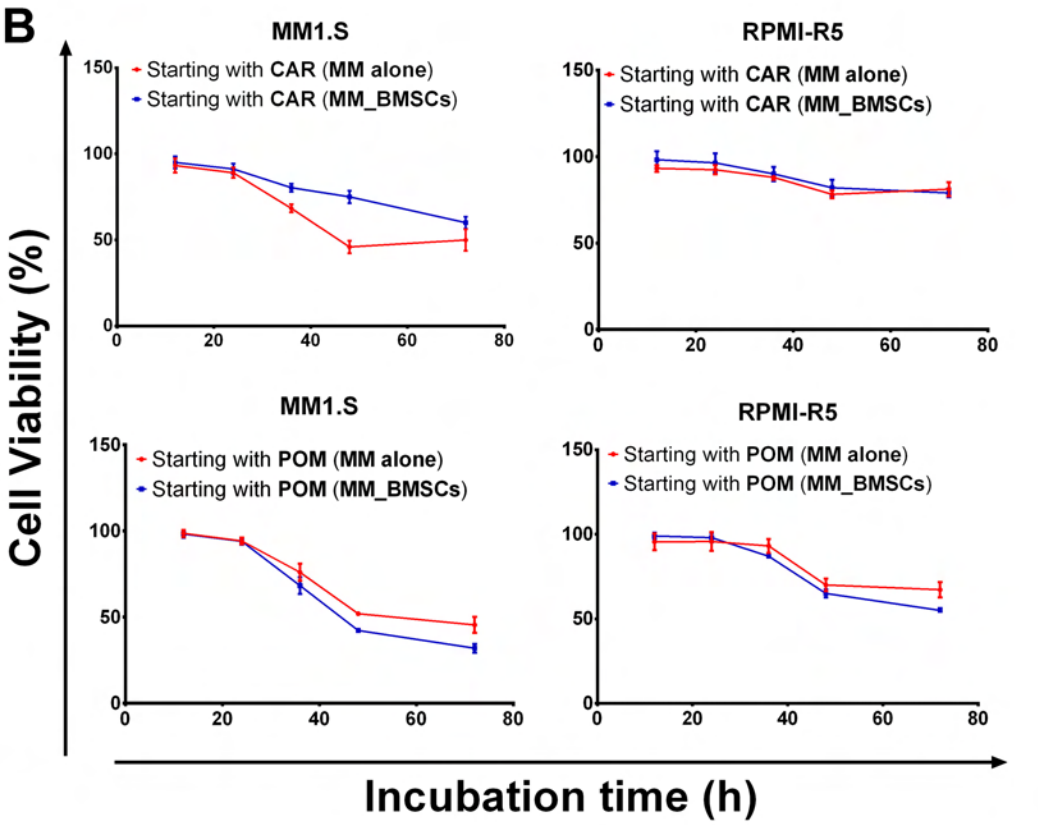
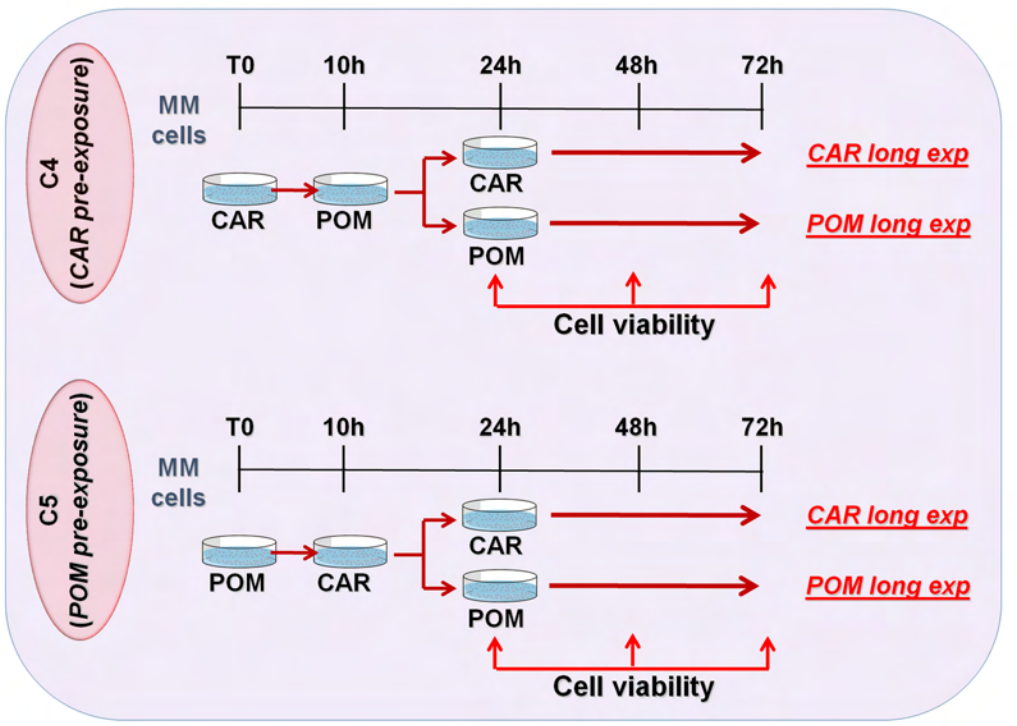

A
RPMI-R5
Dose-escalation with BOR and LEN
24h
48h
Cell Viability (%)
Ctrl
BOR 2 nM
LEN 2 μM
BOR 4 nM
LEN 4 μM
BOR 8 nM
LEN 8 μM
BOR 15 nM
LEN 15 μM
C1
C2
C3
B
RPMI-R5
Dose-escalation with CAR and POM
CAR 2 nM
POM 2 μM
CAR 4 nM
POM 4 μM
CAR 8 nM
POM 8 μM
CAR 15 nM
POM 15 μM
C
24h
48h
RPMI-R5
RPMI-R5_HS-5

Figure 2


A
T0
12h
24h
36h
48h
72h
MM cells
Alternating combo
CAR
POM
CAR
POM
CAR
POM
CAR
POM
CAR
POM
Cell viability


B
MM1.S
Starting with CAR (MM alone)
Starting with CAR (MM_BMSCs)
RPMI-R5
Starting with CAR (MM alone)
Starting with CAR (MM_BMSCs)
MM1.S
Starting with POM (MM alone)
Starting with POM (MM_BMSCs)
RPMI-R5
Starting with POM (MM alone)
Starting with POM (MM_BMSCs)
Cell Viability (%)
Incubation time (h)


C
C4 (CAR pre-exposure)
T0
10h
24h
48h
72h
MM cells
CAR
POM
CAR
POM
CAR long exp
POM long exp
Cell viability
C5 (POM pre-exposure)
T0
10h
24h
48h
72h
MM cells
POM
CAR
CAR
POM
CAR long exp
POM long exp
Cell viability

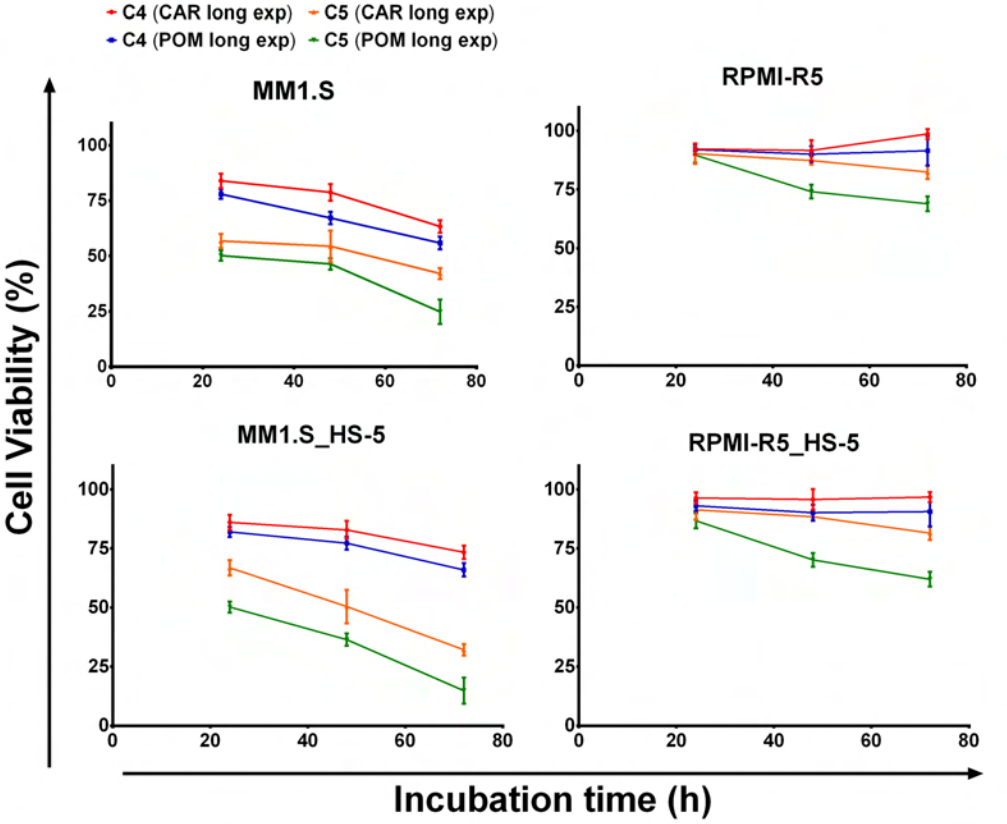

D
C4 (CAR long exp)
C5 (CAR long exp)
C4 (POM long exp)
C5 (POM long exp)
MM1.S
RPMI-R5
MM1.S_HS-5
RPMI-R5_HS-5
Cell Viability (%)
Incubation time (h)

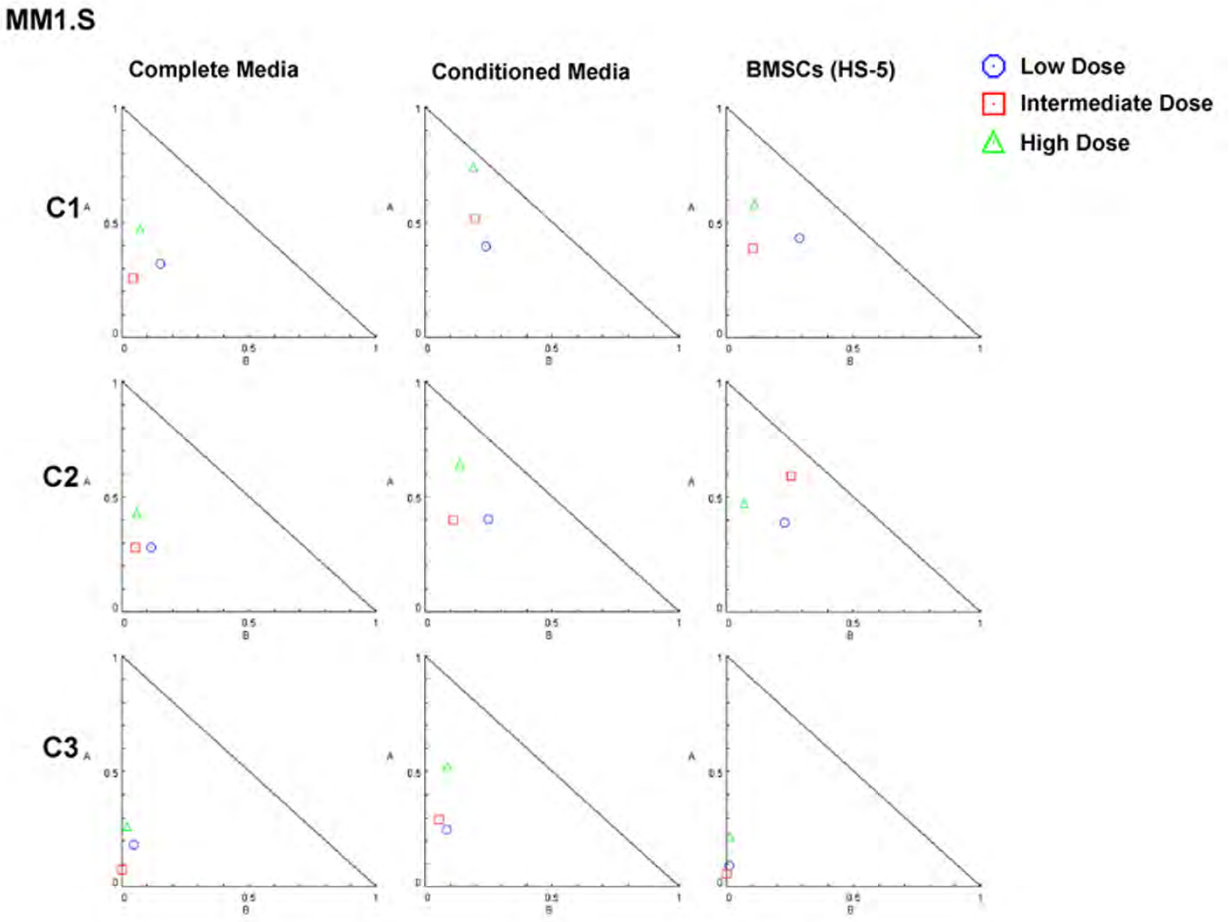
Figure 3

IC_{50} µM	CAR	POM
MM1.S (24h)	*0,00626*	*10,19470*
MM1.S (48h)	*0,00404*	*5,77992*
RPMI-R5 (24h)	*0,020298*	*25,98430*
RPMI-R5 (48h)	*0,104180*	*15,99215*

IC_{50} µM	BOR	LEN
MM1.S (24h)	*0,00718*	*13,00070*
MM1.S (48h)	*0,00572*	*8,35488*
RPMI-R5 (24h)	*0,03049*	*34,13540*
RPMI-R5 (48h)	*0,01639*	*19,06771*

IC_{50} µM	CAR	POM
U266 (24h)	*0,01034*	*17,05520*
U266 (48h)	*0,06362*	*8,97320*
NCI-H929 (24h)	*0,00915*	*10,06819*
NCI-H9292 (48h)	*0,00552*	*5,95379*
KMS12.BM (24h)	*0,01225*	*10,99550*
KMS12.BM (48h)	*0,00881*	*6,92362*
OPM-2 (24h)	*0,01175*	*11,56482*
OPM-2 (48h)	*0,00517*	*6,92362*

Table S1. IC_{50} is the concentration of a drug that gives half-maximal response.



Supplementary S1. MM1.S isobolograms. Isobologram analysis of cytotoxic interaction of CAR and POM under stromal free conditions and in co-culture with HS-5 cells. Cell proliferation was measured by MTT assays and expressed as a percentage of the corresponding untreated cells. Dose response curves of each combination were generated to make non-constant normalized isobolograms. The isobolograms shown are representative of one experiment. Low dose-*blue circle*, intermediate dose-*red square*, high dose-*green triangle.*

Combination Index assessment. The mode of interaction between PIs and IMiDs could be analyzed by the Chou and Talalay method (*Chou TC. Cancer research. 2010 Jan 15;70(2):440-6)*. In order to assess whether the interactions between PIs and IMiDs were synergistic, additive, or antagonistic, combination indexes (CIs) were calculated using CompuSyn software according to the manufacturer's instructions. Briefly, to generate isobolograms, we first obtained the dose-response curves of PIs and IMiDs in combination under each culture condition. Then, we generated non-constant normalized isobolograms and determined the CI values at IC_{50} using CompuSyn software *(Chou TC. Cancer research. 2010 Jan 15;70(2):440-6; Chou TC, Talalay P. Advances in enzyme regulation. 1984;22:27-55*).

MM1.S (24h)

	Experimental condition	*CAR (nM)*	*POM (μM)*	*Simultaneous exposure C1*		*CAR pre-exposure (10h) C2*		*POM pre-exposure (10h) C3*	
				CI	**SD**	**CI**	**SD**	**CI**	**SD**
LOW DOSE	***Complete Media***	2	2	0.513	0.053	0.415	0.022	0.264	0.051
	Co-culture with HS-5	2	2	0.659	0.030	0.647	0.033	0.139	0.048
	Conditioned Media	2	2	0.753	0.039	0.682	0.039	0.362	0.039
INT. DOSE	***Complete Media***	4	4	0.348	0.060	0.382	0.068	0.089	0.016
	Co-culture with HS-5	4	4	0.739	0.030	0.865	0.021	0.073	0.024
	Conditioned Media	4	4	0.543	0.066	0.581	0.098	0.289	0.083
HIGH DOSE	***Complete Media***	8	8	0.589	0.057	0.534	0.065	0.272	0.017
	Co-culture with HS-5	8	8	0.513	0.024	0.594	0.064	0.222	0.017
	Conditioned Media	8	8	0.723	0.039	0.826	0.062	0.587	0.038

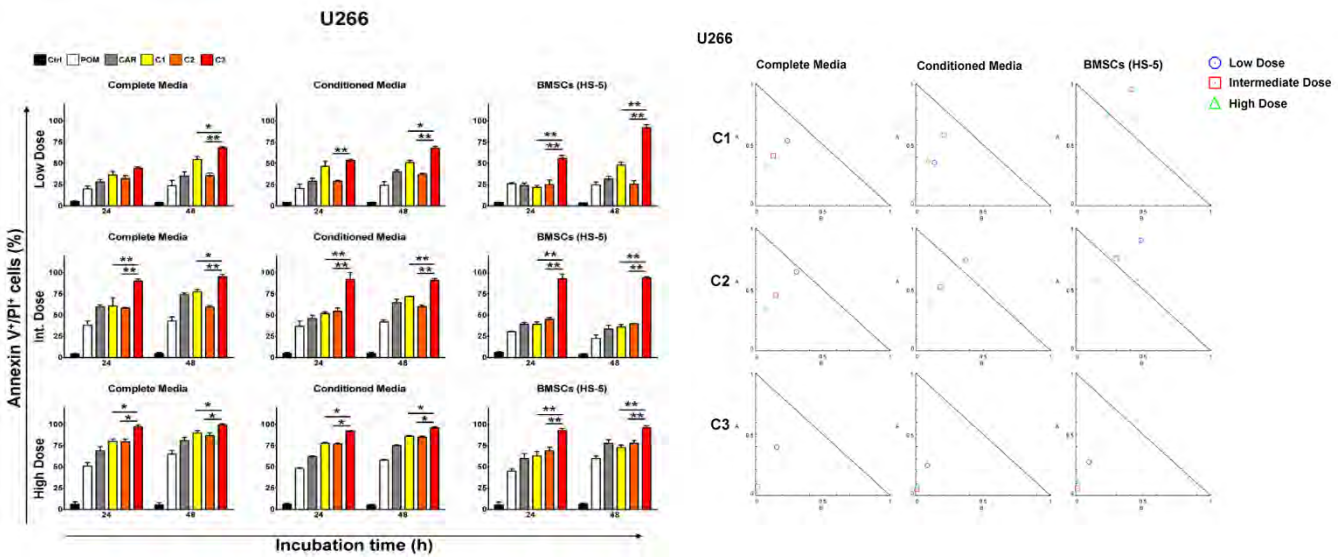
MM1.S (48h)

	Experimental condition	*CAR (nM)*	*POM (μM)*	*Simultaneous exposure C1*		*CAR pre-exposure (10h) C2*		*POM pre-exposure (10h) C3*	
				CI	**SD**	**CI**	**SD**	**CI**	**SD**
LOW DOSE	***Complete Media***	2	2	0.476	0.090	0.404	0.064	0.078	0.002
	Co-culture with HS-5	2	2	0.831	0.070	0.584	0.066	0.065	0.007
	Conditioned Media	2	2	1.484	0.023	1.057	0.061	0.475	0.035
INT. DOSE	***Complete Media***	4	4	0.424	0.065	0.400	0.071	0.095	0.007
	Co-culture with HS-5	4	4	0.751	0.069	0.886	0.020	0.077	0.004
	Conditioned Media	4	4	0.685	0.064	0.654	0.064	0.373	0.047
HIGH DOSE	***Complete Media***	8	8	0.745	0.064	0.572	0.068	0.287	0.021
	Co-culture with HS-5	8	8	0.981	0.002	0.953	0.039	0.135	0.095
	Conditioned Media	8	8	0.866	0.076	0.963	0.053	0.517	0.024

Table S2. Combination index (CI) and standard deviations (SD) for various MM *in vitro* models exposed to CAR and POM in three differing schedules: simultaneous exposure to CAR and POM (C1), CAR 10h prior to POM (C2) and POM 10h prior CAR (C3). Three different dosages of CAR

and POM were used: low dose (2 nM and 2 μM for CAR and POM, respectively), intermediate dose (4 nM and 4 μM for CAR and POM, respectively) and high dose (8 nM and 8 μM for CAR and POM, respectively). All experiments were performed twice in triplicate measurements. CI calculated using CompuSyn software. CI<1 synergistic interaction, CI=1 additive interaction and CI>1 antagonistic interaction (*Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Advances in enzyme regulation. 1984;22:27-55*).

A **B**



Supplementary S2. (A) Apoptotic rate on U266 cell line. Early and late apoptotic events on U266 cells upon exposure to either CAR or POM using as single agent or in combination for up to 48h were measured by flow cytometry. Values shown in histograms are mean ± SD of two independent experiments. * $p<0.05$ and ** $p<0.01$. Control-*black bars*, Pomalidomide-*white bars*, Carfilzomib-*grey bars*, C1-*yellow bars*, C2-*orange bars*, C3-*red bars*. **(B) U266 isobolograms.** Isobologram analysis of cytotoxic interaction of CAR and POM under stromal free conditions and in co-culture with HS-5 cells. Cell proliferation was measured by MTT assays and expressed as a percentage of the corresponding untreated cells. Dose response curves of each combination were generated to make non-constant normalized isobolograms. The isobolograms shown are representative of one experiment. Low dose-*blue circle*, intermediate dose-*red square*, high dose-*green triangle.*

U266 (24h)

	Experimental condition	*CAR (nM)*	*POM (μM)*	*Simultaneous exposure C1*		*CAR pre-exposure (10h) C2*		*POM pre-exposure (10h) C3*	
				CI	**SD**	**CI**	**SD**	**CI**	**SD**
LOW DOSE	***Complete Media***	2	2	0.822	0.068	0.977	0.033	0.502	0.074
	Co-culture with HS-5	2	2	1.806	0.132	1.394	0.008	0.348	0.017
	Conditioned Media	2	2	0.543	0.066	1.156	0.063	0.378	0.003
INT. DOSE	***Complete Media***	4	4	0.591	0.069	0.654	0.065	0.089	0.001
	Co-culture with HS-5	4	4	1.382	0.026	1.128	0.101	0.066	0.006
	Conditioned Media	4	4	0.832	0.054	0.753	0.066	0.075	0.006
HIGH DOSE	***Complete Media***	8	8	0.476	0.090	0.472	0.068	0.048	0.003
	Co-culture with HS-5	8	8	0.996	0.005	0.756	0.005	0.102	0.003
	Conditioned Media	8	8	0.513	0.066	0.537	0.060	0.121	0.029

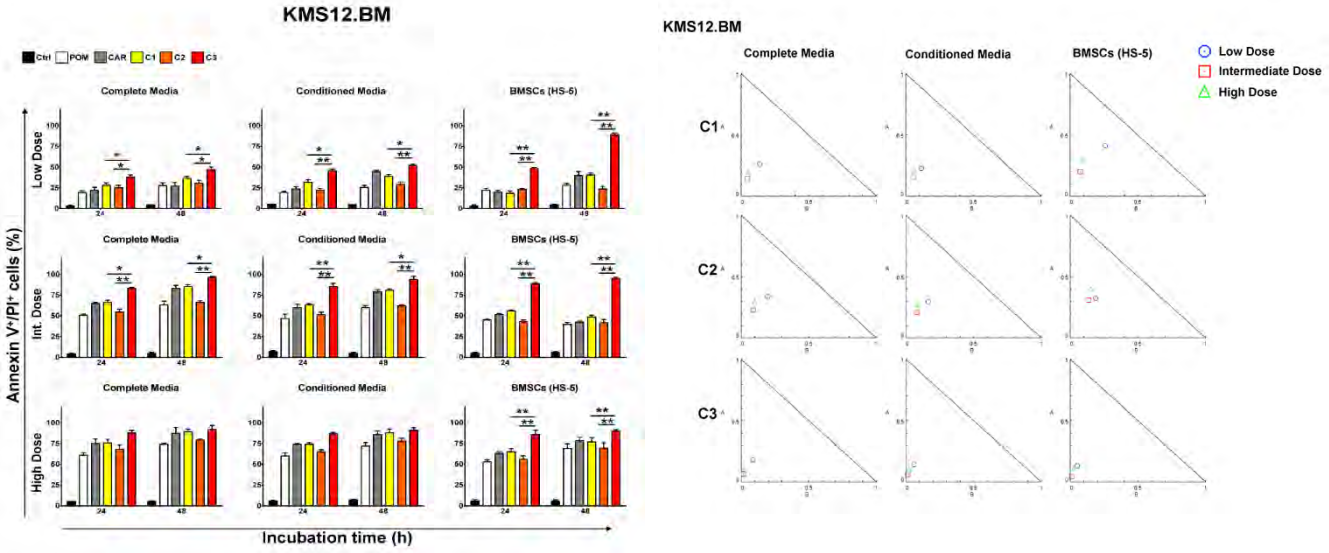
U266 (48h)

	Experimental condition	*CAR (nM)*	*POM (μM)*	*Simultaneous exposure C1*		*CAR pre-exposure (10h) C2*		*POM pre-exposure (10h) C3*	
				CI	**SD**	**CI**	**SD**	**CI**	**SD**
LOW DOSE	***Complete Media***	2	2	0.872	0.068	1.658	0.081	0.438	0.068
	Co-culture with HS-5	2	2	1.076	0.034	2.468	0.379	0.113	0.005
	Conditioned Media	2	2	0.957	0.032	1.452	0.214	0.441	0.071
INT. DOSE	***Complete Media***	4	4	0.681	0.055	1.330	0.042	0.136	0.022
	Co-culture with HS-5	4	4	3.258	0.081	2.608	0.295	0.154	0.041
	Conditioned Media	4	4	0.905	0.105	1.270	0.098	0.236	0.037
HIGH DOSE	***Complete Media***	8	8	0.623	0.066	0.803	0.094	0.046	0.006
	Co-culture with HS-5	8	8	0.970	0.015	1.168	0.045	0.364	0.077
	Conditioned Media	8	8	0.831	0.056	0.913	0.094	0.235	0.035

Table S3. Combination index (CI) and standard deviations (SD) for various MM *in vitro* models exposed to CAR and POM in three differing schedules: simultaneous exposure to CAR and POM (C1), CAR 10h prior to POM (C2) and POM 10h prior CAR (C3). Three different dosages of CAR and POM were used: low dose (2 nM and 2 μM for CAR and POM, respectively), intermediate dose

(4 nM and 4 μM for CAR and POM, respectively) and high dose (8 nM and 8 μM for CAR and POM, respectively). All experiments were performed twice in triplicate measurements. CI calculated using CompuSyn software. CI<1 synergistic interaction, CI=1 additive interaction and CI>1 antagonistic interaction (*Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Advances in enzyme regulation. 1984;22:27-55*).

A **B**



Supplementary S3. (A) Apoptotic rate on KMS12.BM cell line. Early and late apoptotic events on KMS12.BM cells upon exposure to either CAR or POM using as single agent or in combination for up to 48h were measured by flow cytometry. Values shown in histograms are mean ± SD of two independent experiments. * $p<0.05$ and ** $p<0.01$. Control-*black bars*, Pomalidomide-*white bars*, Carfilzomib-*grey bars*, C1-*yellow bars*, C2-*orange bars*, C3-*red bars*. **(B) KMS12.BM isobolograms.** Isobologram analysis of cytotoxic interaction of CAR and POM under stromal free conditions and in co-culture with HS-5 cells. Cell proliferation was measured by MTT assays and expressed as a percentage of the corresponding untreated cells. Dose response curves of each combination were generated to make non-constant normalized isobolograms. The isobolograms shown are representative of one experiment. Low dose-*blue circle*, intermediate dose-*red square*, high dose-*green triangle.*

KMS12.BM (24h)

	Experimental condition	*CAR (nM)*	*POM (μM)*	*Simultaneous exposure C1*		*CAR pre-exposure (10h) C2*		*POM pre-exposure (10h) C3*	
				CI	**SD**	**CI**	**SD**	**CI**	**SD**
LOW DOSE	***Complete Media***	2	2	0.451	0.069	0.510	0.070	0.250	0.029
	Co-culture with HS-5	2	2	0.718	0.060	0.561	0.069	0.153	0.047
	Conditioned Media	2	2	0.398	0.073	0.590	0.071	0.217	0.018
INT. DOSE	***Complete Media***	4	4	0.201	0.013	0.340	0.070	0.088	0.003
	Co-culture with HS-5	4	4	0.330	0.071	0.493	0.067	0.063	0.009
	Conditioned Media	4	4	0.218	0.003	0.371	0.069	0.082	0.011
HIGH DOSE	***Complete Media***	8	8	0.302	0.068	0.404	0.065	0.122	0.001
	Co-culture with HS-5	8	8	0.450	0.070	0.603	0.067	0.170	0.042
	Conditioned Media	8	8	0.323	0.067	0.450	0.070	0.166	0.048

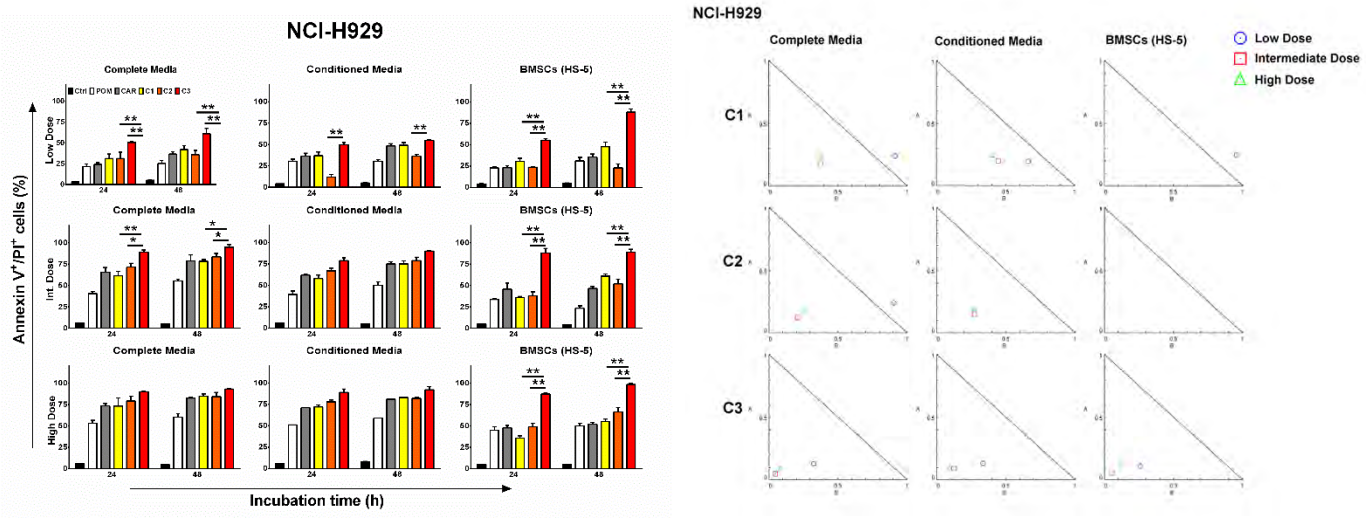
KMS12.BM (48h)

	Experimental condition	*CAR (nM)*	*POM (μM)*	*Simultaneous exposure C1*		*CAR pre-exposure (10h) C2*		*POM pre-exposure (10h) C3*	
				CI	**SD**	**CI**	**SD**	**CI**	**SD**
LOW DOSE	***Complete Media***	2	2	0.511	0.070	0.621	0.069	0.374	0.065
	Co-culture with HS-5	2	2	0.451	0.069	0.811	0.070	0.059	0.002
	Conditioned Media	2	2	0.474	0.065	0.651	0.069	0.194	0.119
INT. DOSE	***Complete Media***	4	4	0.178	0.045	0.392	0.068	0.046	0.005
	Co-culture with HS-5	4	4	0.664	0.065	0.784	0.022	0.042	0.018
	Conditioned Media	4	4	0.243	0.080	0.451	0.070	0.053	0.019
HIGH DOSE	***Complete Media***	8	8	0.271	0.069	0.461	0.069	0.139	0.041
	Co-culture with HS-5	8	8	0.450	0.070	0.664	0.066	0.168	0.054
	Conditioned Media	8	8	0.291	0.069	0.481	0.069	0.169	0.027

Table S4. Combination index (CI) and standard deviations (SD) for various MM in vitro models exposed to CAR and POM in three differing schedules: simultaneous exposure to CAR and POM

(C1), CAR 10h prior to POM (C2) and POM 10h prior CAR (C3). Three different dosages of CAR and POM were used: low dose (2 nM and 2 μM for CAR and POM, respectively), intermediate dose (4 nM and 4 μM for CAR and POM, respectively) and high dose (8 nM and 8 μM for CAR and POM, respectively). All experiments were performed twice in triplicate measurements. CI calculated using CompuSyn software. CI<1 synergistic interaction, CI=1 additive interaction and CI>1 antagonistic interaction (*Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Advances in enzyme regulation. 1984;22:27-55*).

A **B**



Supplementary S4. (A) Apoptotic rate on NCI-H929 cell line. Early and late apoptotic events on NCI-H929 cells upon exposure to either CAR or POM using as single agent or in combination for up to 48h were measured by flow cytometry. Values shown in histograms are mean ± SD of two independent experiments. * $p<0.05$ and ** $p<0.01$. Control-*black bars*, Pomalidomide-*white bars*, Carfilzomib-*grey bars*, C1-*yellow bars*, C2-*orange bars*, C3-*red bars*. **(B) NCI-H929 isobolograms.** Isobologram analysis of cytotoxic interaction of CAR and POM under stromal free conditions and in co-culture with HS-5 cells. Cell proliferation was measured by MTT assays and expressed as a percentage of the corresponding untreated cells. Dose response curves of each combination were generated to make non-constant normalized isobolograms. The isobolograms shown are representative of one experiment. Low dose-*blue circle*, intermediate dose-*red square*, high dose-*green triangle*.

NCI.H929 (24h)

	Experimental condition	*CAR (nM)*	*POM (μM)*	*Simultaneous exposure C1*		*CAR pre-exposure (10h) C2*		*POM pre-exposure (10h) C3*	
				CI	**SD**	**CI**	**SD**	**CI**	**SD**
LOW DOSE	***Complete Media***	2	2	1.137	0.024	1.174	0.037	0.476	0.034
	Co-culture with HS-5	2	2	1.251	0.069	1.906	0.133	0.385	0.021
	Conditioned Media	2	2	0.882	0.025	4.398	0.591	0.484	0.023
INT. DOSE	***Complete Media***	4	4	0.603	0.066	0.393	0.081	0.099	0.001
	Co-culture with HS-5	4	4	1.860	0.057	1.720	0.114	0.154	0.065
	Conditioned Media	4	4	0.694	0.065	0.482	0.083	0.224	0.009
HIGH DOSE	***Complete Media***	8	8	0.664	0.068	0.487	0.074	0.188	0.017
	Co-culture with HS-5	8	8	3.488	0.266	1.924	0.062	0.218	0.025
	Conditioned Media	8	8	0.702	0.068	0.502	0.054	0.198	0.003

NCI.H929 (48h)

	Experimental condition	*CAR (nM)*	*POM (μM)*	*Simultaneous exposure C1*		*CAR pre-exposure (10h) C2*		*POM pre-exposure (10h) C3*	
				CI	**SD**	**CI**	**SD**	**CI**	**SD**
LOW DOSE	***Complete Media***	2	2	0.858	0.060	1.159	0.057	0.284	0.076
	Co-culture with HS-5	2	2	0.673	0.067	2.286	0.264	0.047	0.025
	Conditioned Media	2	2	0.634	0.065	1.097	0.004	0.426	0.037
INT. DOSE	***Complete Media***	4	4	0.321	0.069	0.211	0.028	0.056	0.005
	Co-culture with HS-5	4	4	0.722	0.068	1.061	0.055	0.114	0.006
	Conditioned Media	4	4	0.381	0.070	0.295	0.049	0.108	0.003
HIGH DOSE	***Complete Media***	8	8	0.386	0.062	0.414	0.065	0.122	0.031
	Co-culture with HS-5	8	8	1.641	0.199	1.076	0.035	0.036	0.005
	Conditioned Media	8	8	0.443	0.066	0.473	0.066	0.138	0.040

Table S5. Combination index (CI) and standard deviations (SD) for various MM in vitro models exposed to CAR and POM in three differing schedules: simultaneous exposure to CAR and POM

(C1), CAR 10h prior to POM (C2) and POM 10h prior CAR (C3). Three different dosages of CAR and POM were used: low dose (2 nM and 2 μM for CAR and POM, respectively), intermediate dose (4 nM and 4 μM for CAR and POM, respectively) and high dose (8 nM and 8 μM for CAR and POM, respectively). All experiments were performed twice in triplicate measurements. CI calculated using CompuSyn software. CI<1 synergistic interaction, CI=1 additive interaction and CI>1 antagonistic interaction (*Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Advances in enzyme regulation. 1984;22:27-55*).

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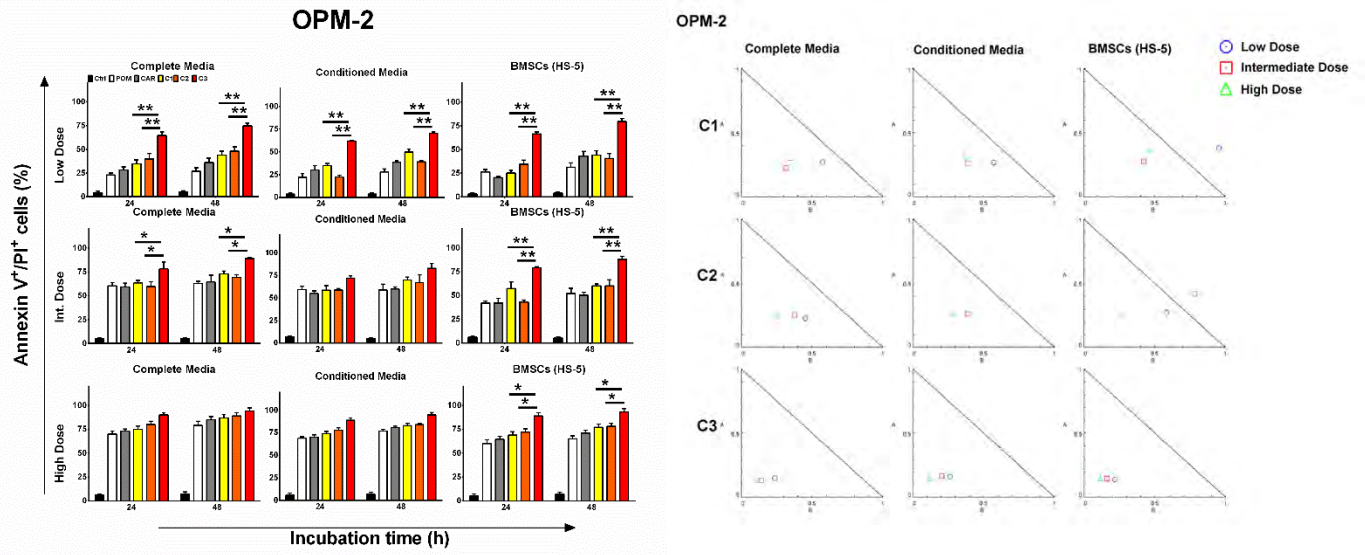


Supplementary S5. (A) Apoptotic rate on OPM-2 cell line. Early and late apoptotic events on OPM-2 cells upon exposure to either CAR or POM using as single agent or in combination for up to 48h were measured by flow cytometry. Values shown in histograms are mean ± SD of two independent experiments. * $p<0.05$ and ** $p<0.01$. Control-*black bars*, Pomalidomide-*white bars*, Carfilzomib-*grey bars*, C1-*yellow bars*, C2-*orange bars*, C3-*red bars*. **(B) OPM-2 isobolograms.** Isobologram analysis of cytotoxic interaction of CAR and POM under stromal free conditions and in co-culture with HS-5 cells. Cell proliferation was measured by MTT assays and expressed as a percentage of the corresponding untreated cells. Dose response curves of each combination were generated to make non-constant normalized isobolograms. The isobolograms shown are representative of one experiment. Low dose-*blue circle*, intermediate dose-*red square*, high dose-*green triangle.*

OPM-2 (24h)

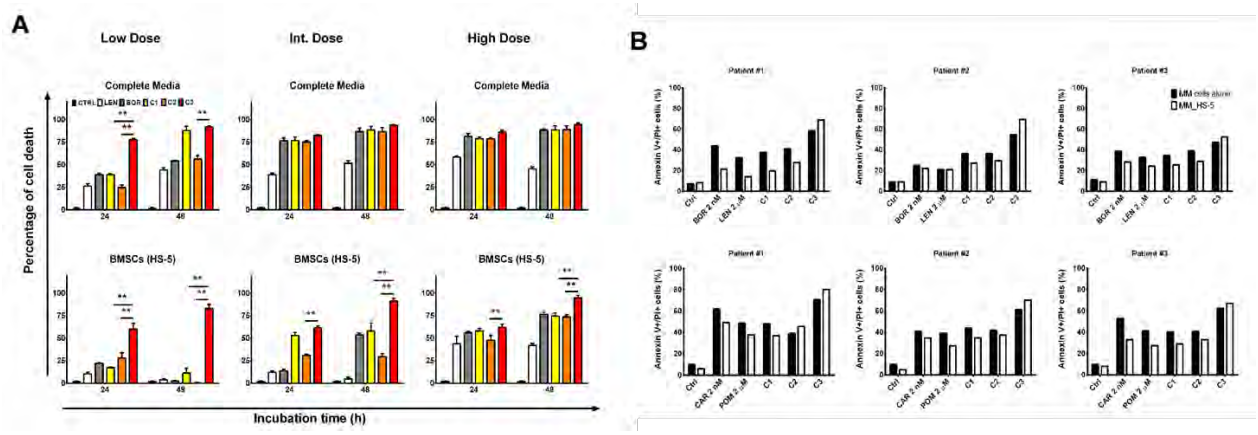
	Experimental condition	*CAR (nM)*	*POM (μM)*	*Simultaneous exposure C1*		*CAR pre-exposure (10h) C2*		*POM pre-exposure (10h) C3*	
				CI	**SD**	**CI**	**SD**	**CI**	**SD**
LOW DOSE	***Complete Media***	2	2	0.864	0.022	0.692	0.011	0.393	0.010
	Co-culture with HS-5	2	2	1.369	0.044	0.869	0.016	0.355	0.007
	Conditioned Media	2	2	0.861	0.027	1.581	0.027	0.453	0.039
INT. DOSE	***Complete Media***	4	4	0.562	0.026	0.661	0.040	0.288	0.017
	Co-culture with HS-5	4	4	0.740	0.056	1.218	0.017	0.314	0.008
	Conditioned Media	4	4	0.673	0.024	0.675	0.035	0.387	0.018
HIGH DOSE	***Complete Media***	8	8	0.655	0.008	0.519	0.030	0.269	0.044
	Co-culture with HS-5	8	8	0.830	0.013	0.548	0.031	0.286	0.034
	Conditioned Media	8	8	0.701	0.027	0.578	0.031	0.286	0.034

OPM-2 (48h)

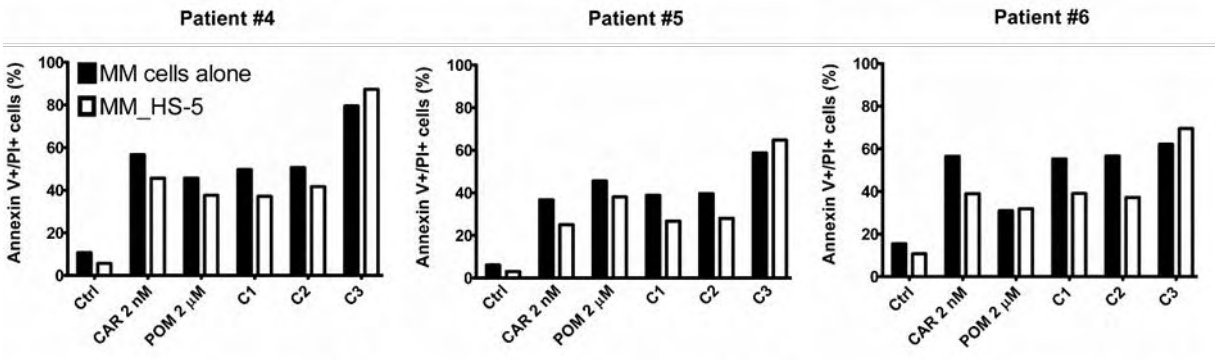
	Experimental condition	*CAR (nM)*	*POM (μM)*	*Simultaneous exposure C1*		*CAR pre-exposure (10h) C2*		*POM pre-exposure (10h) C3*	
				CI	**SD**	**CI**	**SD**	**CI**	**SD**
LOW DOSE	***Complete Media***	2	2	0.794	0.108	0.661	0.070	0.272	0.059
	Co-culture with HS-5	2	2	0.744	0.037	0.870	0.057	0.139	0.027
	Conditioned Media	2	2	0.609	0.059	0.932	0.068	0.299	0.098
INT. DOSE	***Complete Media***	4	4	0.391	0.070	0.561	0.069	0.144	0.034
	Co-culture with HS-5	4	4	0.802	0.067	0.804	0.065	0.146	0.051
	Conditioned Media	4	4	0.543	0.066	0.613	0.067	0.234	0.034
HIGH DOSE	***Complete Media***	8	8	0.442	0.068	0.383	0.067	0.152	0.060
	Co-culture with HS-5	8	8	0.694	0.066	0.694	0.066	0.216	0.009
	Conditioned Media	8	8	0.563	0.067	0.517	0.046	0.138	0.040

Table S6. Combination index (CI) and standard deviations (SD) for various MM in vitro models exposed to CAR and POM in three differing schedules: simultaneous exposure to CAR and POM (C1), CAR 10h prior to POM (C2) and POM 10h prior CAR (C3). Three different dosages of CAR

and POM were used: low dose (2 nM and 2 μM for CAR and POM, respectively), intermediate dose (4 nM and 4 μM for CAR and POM, respectively) and high dose (8 nM and 8 μM for CAR and POM, respectively). All experiments were performed twice in triplicate measurements. CI calculated using CompuSyn software. CI<1 synergistic interaction, CI=1 additive interaction and CI>1 antagonistic interaction (*Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Advances in enzyme regulation. 1984;22:27-55*).



Supplementary S6. (A) MTT assay of response of MM1.S to BOR and LEN combo. Three different drug concentrations were used for each agent: *low dose* (BOR 2nM and LEN 2 μM, respectively), *intermediate dose* (BOR 4 nM and LEN 4 μM, respectively) and *high dose* (BOR 8 nM and LEN 8 μM, respectively). Cells were treated with or without BMSCs. Control-*black bars*, Lenalidomide-*white bars*, Bortezomib-*grey bars*, C1-*yellow bars*, C2-*orange bars*, C3-*red bars*. Values shown in histograms are mean ± SD of two independent experiments. ** $p<0.01$. **(B) Flow cytometry analysis of CD138+ cells derived from newly diagnosis MM patients.** Three MM patients were treated in the presence or absence of HS-5 cells with low dosage of PIs and IMiDs as previously described (BOR/CAR 2nM and LEN/POM 2 μM, respectively). Flow cytometry analysis showed a higher apoptotic rate when cells were treated with IMiDs prior to PIs in all patients tested. This effect was more pronounce in the presence of BMSCs. MM cells alone-*black bars*, MM cells co-coltured with HS-5-*white bars*.



	MM cells alone (%)	MM_HS-5 (%)
CTRL	10.00	5.00
CAR	56.00	45.00
POM	45.00	37.00
C1	49.00	36.50
C2	50.00	41.00
C3	78.90	86.70

	MM cells alone (%)	MM_HS-5 (%)
CTRL	6.00	3.00
CAR	36.70	25.00
POM	45.60	38.00
C1	38.80	26.70
C2	39.60	28.00
C3	58.70	64.70

	MM cells alone (%)	MM_HS-5 (%)
CTRL	14.83	10.11
CAR	55.90	38.31
POM	30.30	31.30
C1	54.60	38.40
C2	56.00	36.58
C3	61.50	68.90

Supplementary S7. Flow cytometry analysis of CD138+ cells derived from newly diagnosis MM patients. Additional three MM patients were treated in the presence or absence of HS-5 cells with low dosage of CAR and POM as previously described (CAR 2nM and POM 2 μM, respectively). Flow cytometry analysis showed a higher apoptotic rate when cells were treated with POM prior to CAR in all patients tested. This effect was more pronounce in the presence of BMSCs. MM cells alone-*black bars*, MM cells co-coltured with HS-5-*white bars*.