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

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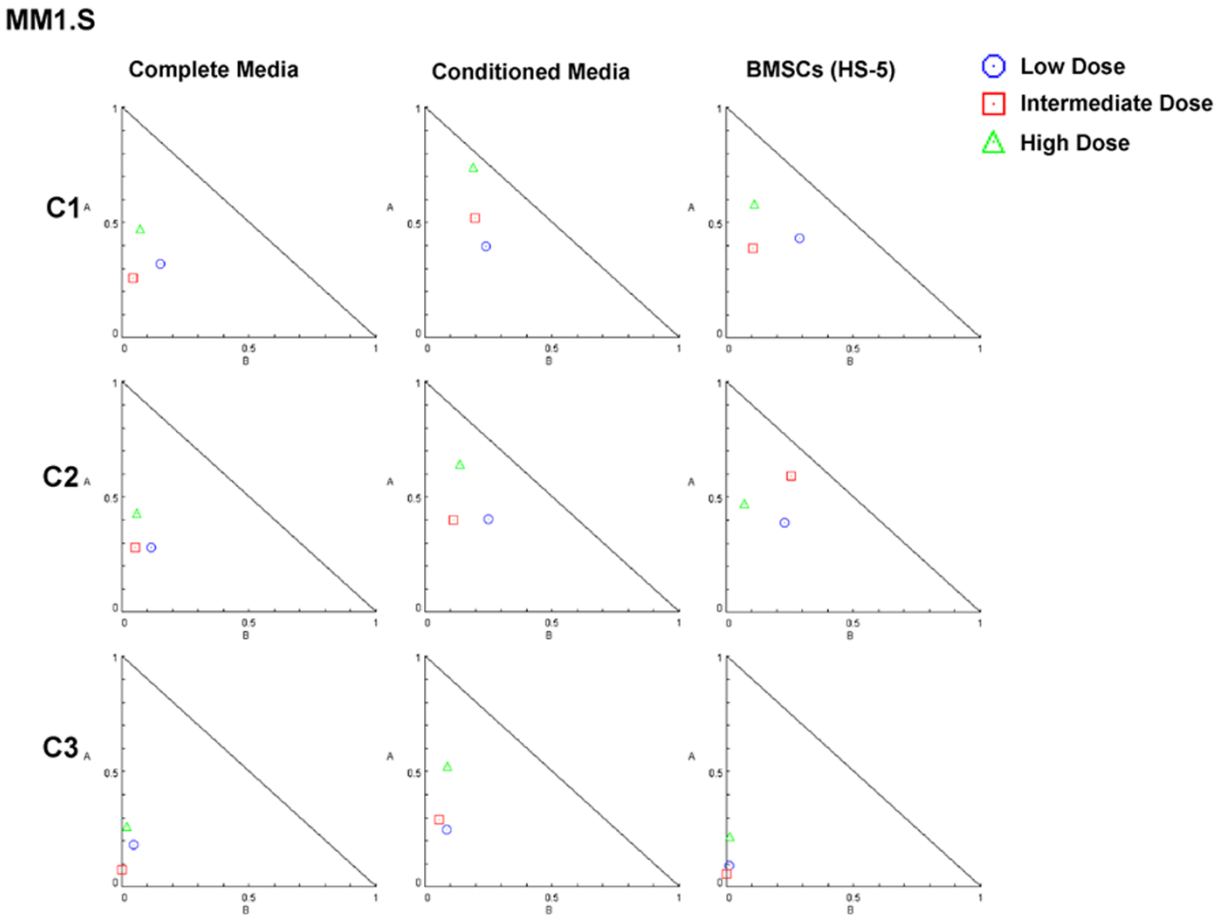
IC_{50} µM	CAR	POM
MM1.S (24h)	*0,00626*	*10,19470*
MM1.S (48h)	*0,00404*	*5,77992*
RPMI-R5 (24h)	*0,020298*	*25,98430*
RPMI-R5 (48h)	*0,104180*	*15,99215*

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*).

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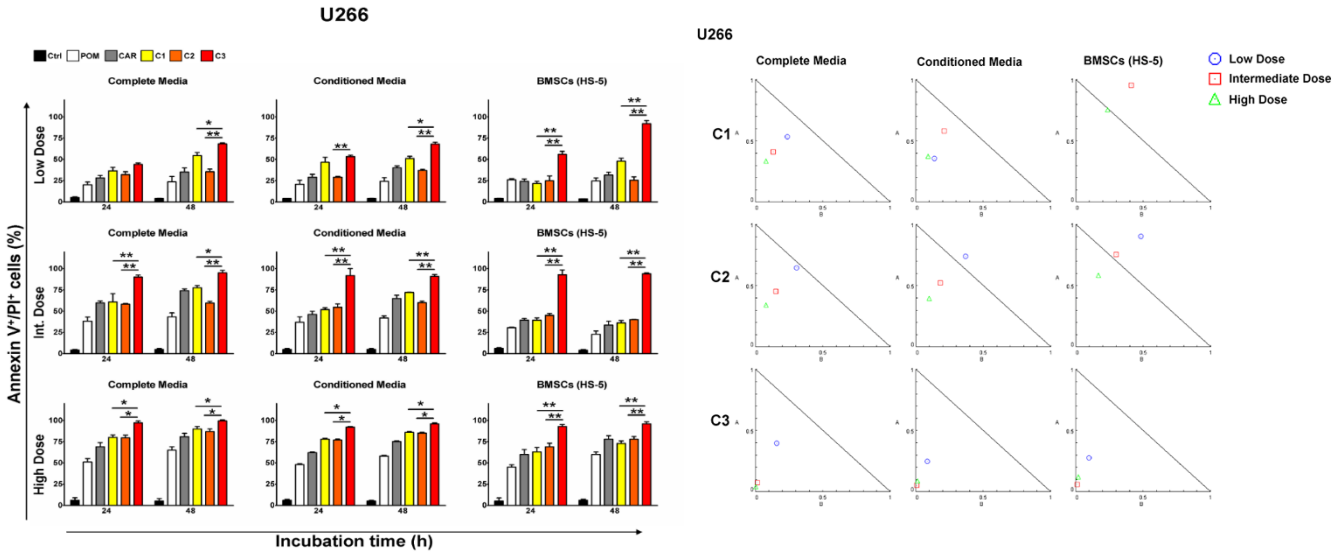

Supplementary S2. (A) Apoptotic rate on U266 cell line. Early and late apoptotic events on U266 cells upon exposure to either CAR or POM using as single agent or in combination for up to 48h were measured by flow cytometry. Values shown in histograms are mean ± SD of two independent experiments. * $p<0.05$ 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*).

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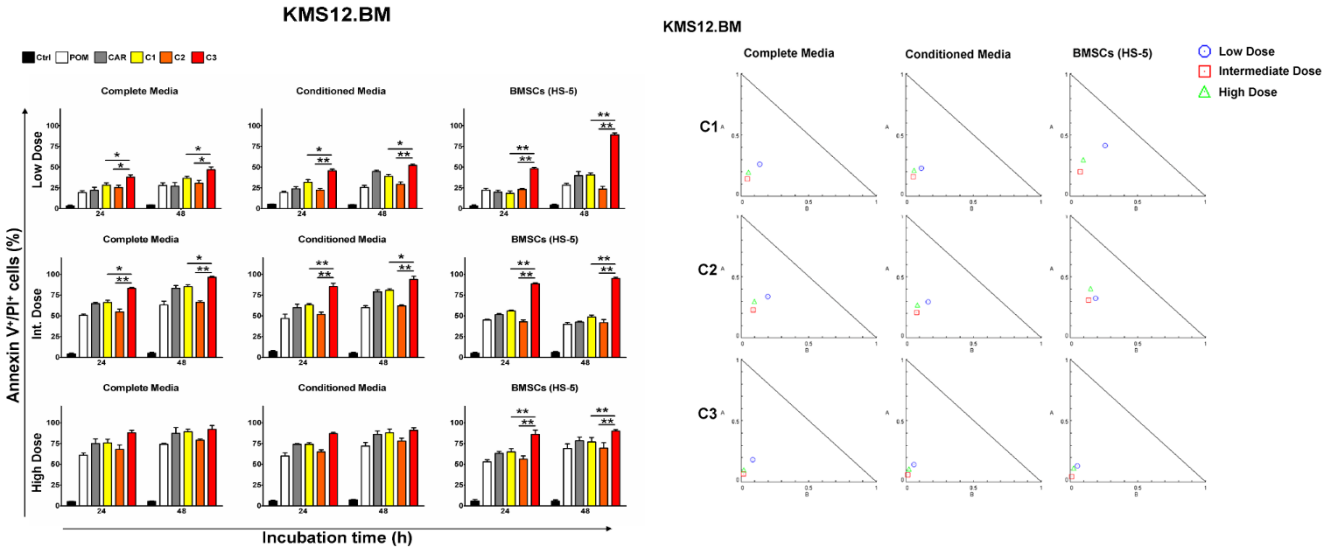

Supplementary S3. (A) Apoptotic rate on KMS12.BM cell line. Early and late apoptotic events on KMS12.BM cells upon exposure to either CAR or POM using as single agent or in combination for up to 48h were measured by flow cytometry. Values shown in histograms are mean ± SD of two independent experiments. * $p<0.05$ 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*).

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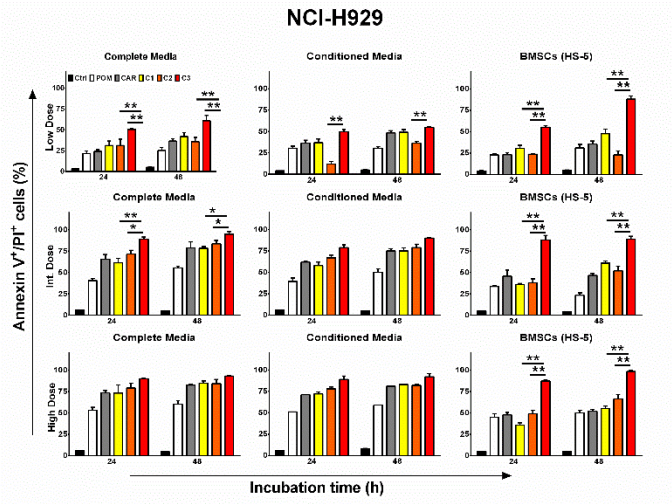

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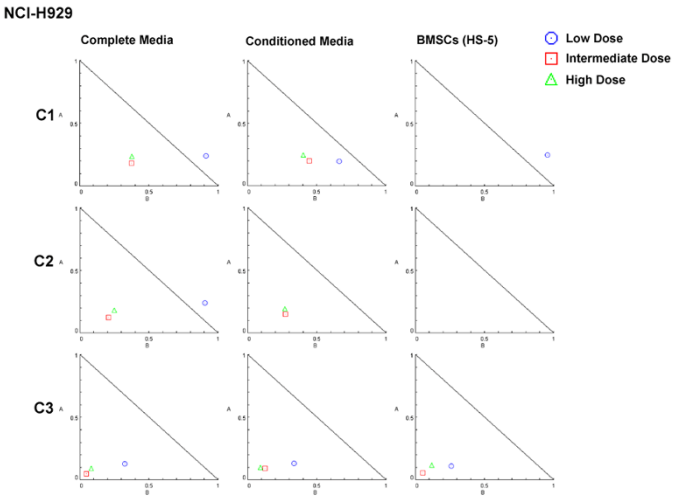

Supplementary S4. (A) Apoptotic rate on NCI-H929 cell line. Early and late apoptotic events on NCI-H929 cells upon exposure to either CAR or POM using as single agent or in combination for up to 48h were measured by flow cytometry. Values shown in histograms are mean ± SD of two independent experiments. * $p<0.05$ 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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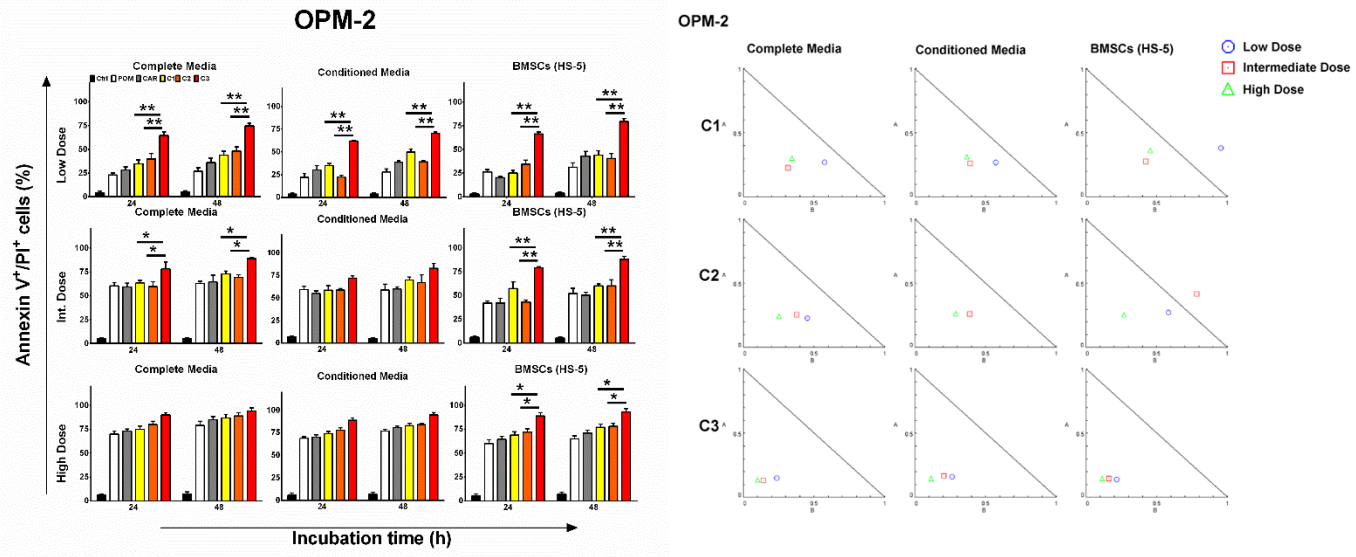


Supplementary S5. (A) Apoptotic rate on OPM-2 cell line. Early and late apoptotic events on OPM-2 cells upon exposure to either CAR or POM using as single agent or in combination for up to 48h were measured by flow cytometry. Values shown in histograms are mean ± SD of two independent experiments. * $p<0.05$ 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)

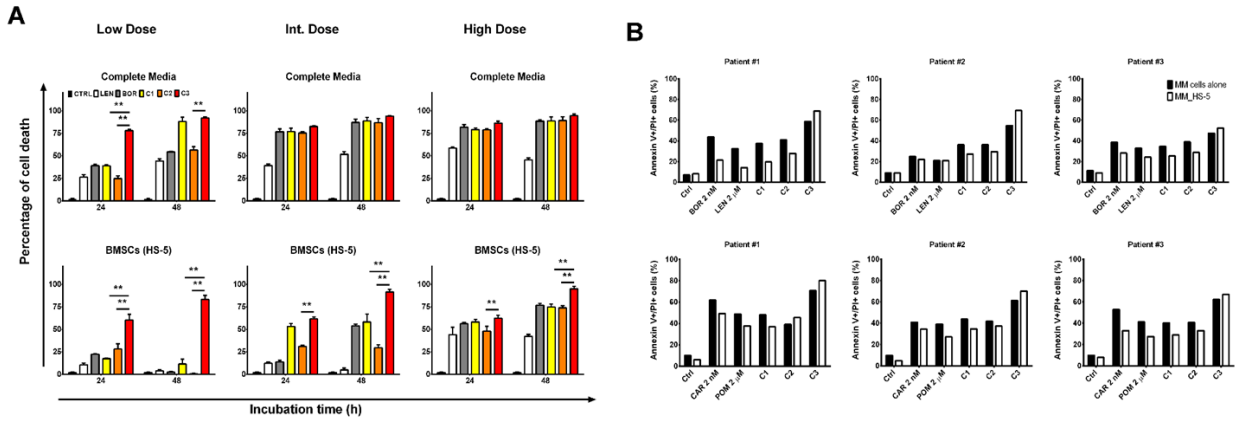
	Experimental condition	*CAR (nM)*	*POM (μM)*	*Simultaneous exposure C1*		*CAR pre-exposure (10h) C2*		*POM pre-exposure (10h) C3*	
				CI	**SD**	**CI**	**SD**	**CI**	**SD**
LOW DOSE	***Complete Media***	2	2	0.864	0.022	0.692	0.011	0.393	0.010
	Co-culture with HS-5	2	2	1.369	0.044	0.869	0.016	0.355	0.007
	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)

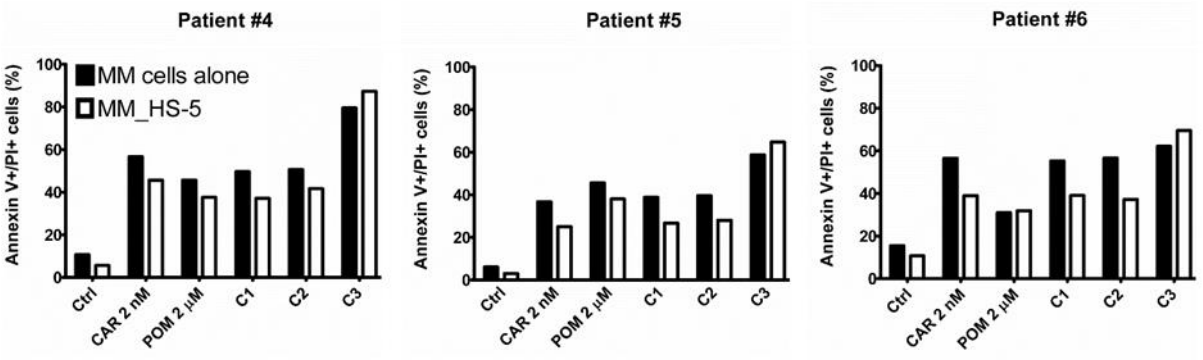
	Experimental condition	*CAR (nM)*	*POM (μM)*	*Simultaneous exposure C1*		*CAR pre-exposure (10h) C2*		*POM pre-exposure (10h) C3*	
				CI	**SD**	**CI**	**SD**	**CI**	**SD**
LOW DOSE	***Complete Media***	2	2	0.794	0.108	0.661	0.070	0.272	0.059
	Co-culture with HS-5	2	2	0.744	0.037	0.870	0.057	0.139	0.027
	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*.