Possibilities and limitations of an *in vitro* three-dimensional bone marrow model for the prediction of clinical responses in patients with relapsed multiple myeloma

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Supplemental Data

Methods

Primary tissue collection

The use of cord blood, healthy BM samples (of orthopedic patients undergoing hip replacement surgery) and MM patient BM samples was approved by the local ethics committee of the University Medical Center Utrecht (METC number 01-230, 08-001 and 09-265). Also the comparison of study outcomes to coded clinical data (treatment history, clinical treatment outcomes and clinically determined cytogenetics) was approved by the local ethics committee (TCBio number 16-088). All samples were obtained after written informed consent. The clinical data were collected from the electronic patient files that collect all baseline information, given treatments, and laboratory measurements such as M-protein and cytogenetic data. In the relapsed setting, fluorescence in situ hybridization cytogenetic testing is mostly restricted to known previous cytogenetic abnormalities and in addition 1p, 1q and 17p abnormalities.

Cell line and primary cell culture

Human myeloma cell lines OPM2 and L363 were obtained from the American Type Culture Collection and cultured for 2 passages before experimental use. No mycoplasma testing was performed. Primary $CD138^+$ cells were isolated from cryopreserved mononuclear cells (MNCs) originating from MM BM. Isolation was performed using $CD138^+$ human microbeads (Miltenyi Biotec, Germany) resulting in varying levels of $CD138^+$ purity (Figure 1C). Myeloma cells were cultured in advanced RPMI 1640 medium, 10% (v/v) fetal bovine serum (FBS), 2 mM of L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (all Gibco, ThermoFisher, USA). Multipotent mesenchymal stromal cells (MSCs) and endothelial progenitor cells (EPCs) were isolated previously from healthy BM and cord blood respectively.[1, 2] MSCs were expanded in MSC-medium (α-minimal essential media (αMEM, Gibco, USA),10% (v/v) FBS, 0.2 mM L-ascorbic acid 2-phosphate, 100 U/ml penicillin and 100 μg/ml streptomycin), EPCs were seeded on collagen I (BD Biosciences)-coated wells and expanded in EGM-2 medium (Lonza, Switzerland), SingleQuots™ Kit (Lonza, Switzerland), 10% (v/v) FBS, 100 U/ml penicillin and 100 μg/ml streptomycin).

3D bone marrow-multiple myeloma model

All 3D co-cultures were performed as described previously.[1] In short, cells were cultured at 37°C in growth factor-reduced Matrigel (Corning, USA) diluted by an equal volume of α-MEM. For cell labelling, DiD (Vybrant Multicolor Cell-Labeling Kit, ThermoFisher) was used according to manufacturer’s protocol. MSCs, EPCs and myeloma cells were mixed in a 4:1:1 ratio. Cultures were maintained in mixed medium (containing equal amounts of MSC medium, EPC medium and MM medium) which was changed twice a week.

In vitro drug sensitivity and resistance testing

3D cell line cultures

OPM2 and L363 cells are cultured in 2D, or co-cultured in 3D with MSCs and EPCs (2D and 3D cultures contained equal myeloma cell numbers). All cells were precultured for 7 days before treatment testing, which was previously shown to induce CAM-DR in the 3D model.[3] Drug sensitivity and resistance testing was performed using oncologic drugs, which the patients had received: lenalidomide (Santa Cruz Biotechnology), pomalidomide (Selleck Chemicals), thalidomide (Selleck Chemicals), bortezomib (Selleck Chemicals), carfilzomib (Cayman Chemical Company), melphalan (Cayman Chemical Company), dexamethasone (D8893 Sigma-Aldrich) and cyclophosphamide. Cyclophosphamide requires activation for *in vitro* studies.[4] Therefore, the active metabolite of cyclophosphamide, 4-hydroperoxy-cyclophosphamide (4HC, Cayman Chemical Company) was used for *in vitro* cyclophosphamide evaluation. Drugs were tested in 2 concentrations; 2 µM and 4 µM (lenalidomide, pomalidomide, thalidomide and dexamethasone), 10 nM and 20 nM (bortezomib, carfilzomib) or 10 µM and 20 µM (melphalan, 4HC) that are referred to as 1x and 2x dose. Drug sensitivity and resistance was analyzed using both flow cytometry (2D and 3D) and confocal imaging (3D) after 72 hours of treatment. To image cell viability, both calcein and ethidium homodimer-1 were added to the 3D plugs (Live/Dead Viability/Cytotoxicity Kit for mammalian cells, ThermoFisher) according to the manufacturer's protocol.

3D primary myeloma cultures

DiD labelled CD138$^+$ myeloma cells (n=7) were co-cultured in 3D with MSCs and EPCs for 14 days, to allow the formation of primary myeloma aggregates within the cultures.[1] Drug sensitivity and resistance testing was performed using the same oncologic drugs as described above. Carfilzomib was tested on the CD138$^+$ myeloma cells of 4 donors, all other drugs were test on the CD138$^+$ myeloma cells of 7 donors. Drugs were tested in 2 concentrations, as described above, which are referred to as 1x and 2x dose. Drug sensitivity and resistance was analyzed using confocal imaging after 72 hours of treatment, after calcein and ethidium homodimer-1 addition for visualization of cell viability.

Flow cytometric analysis

The non-depleted BM-MNCs, CD138$^+$ and CD138$^-$ cell fractions of each MM BM donor were analyzed to determine the purity of the obtained CD138$^+$ fraction after MACS. The cytotoxic activity of the added drugs in 2D and 3D was analyzed for both L363 and OPM2 using flow cytometry. The 3D Matrigel plugs were dissolved using cell recovery solution (Corning), according to the manufacturer's protocol. All conditions were labeled with anti-hCD138-PE (1:100, Biolegend) in the presence of FcR-blocking reagent (Miltenyi). Antibody labeling was performed for 30 min at 4°C, followed by two PBS washing steps. DAPI (100 ng/mL, Biolegend) was added to determine cell death. Flow cytometry analysis was performed using a Becton Dickinson FACS Canto II. Up to 10^4 cells were acquired and analyzed in each sample.

Confocal imaging

All fluorescence images were taken with a Leica SP8X Laser Scanning Confocal Microscope using a white light laser (470-670 nm) and Leica LASX acquisition software. The cytotoxic activity of the drugs within the 3D model was live imaged: hybrid detectors collected fluorescence signal from either calcein (494/500-525) and ethidium homodimer-1 (528/600-640) or calcein (494/500-525), ethidium homodimer-1 (528/600-640) and DiD (644/665-705). Calcein was given the pseudocolor green, ethidium homodimer-1 the pseudocolor red and DiD the pseudocolor blue. Overlays of the channels green and red appear yellow in the obtained images, overlays of the channels blue and green appear cyan, and overlays of the channels blue and red appear magenta.

Overview images of the 3D cultures were made using the mosaic function of the Leica LASX software, stitching the images together using smooth and linear blending. All z-stack images were processed using ImageJ 1.51h software to create single maximum projections.

Treatment outcome analysis

Strict and extended clinical treatment responses

The strict clinical treatment responses include the treatment response to the last clinical therapy before BM aspiration (of which the cells are used in the 3D model), and the treatment response to the therapy given immediately after BM aspiration were compared to the *in vitro* treatment responses.

The extended clinical treatment responses include the treatment responses to all clinical therapies before BM aspiration, and the treatment responses to all therapies given after BM aspiration were compared to the *in vitro* treatment responses.

Clinical treatment outcomes

Progressive disease was defined as the increase in M-protein during therapy (> 25% with a minimum of 5 gr/L). Stable disease was defined as absence of disease progression, and absence of partial response. Partial response was defined as a decrease in M protein during therapy (> 50%). Since in the RRMM also achieving stable disease can be important, both stable disease and partial response were classified as responding to therapy and progressive disease as not responding to therapy.

In vitro treatment outcomes

The cytotoxic activity of the given drugs to the 3D myeloma culture was analyzed by determining the % of dead myeloma cells after treatment, or alternatively, by the number of remaining living myeloma cells after treatment (both compared to the non-treated vehicle control). Progressive disease was defined as <33% of dead myeloma cells, or alternatively a minimal decrease (< 33%) or increase in viable myeloma cells. Partial response was defined as >66% of dead myeloma cells or a >66% drop in the number of living myeloma cells. Stable disease was defined as no progressive disease, but also no partial response. Progressive disease was classified as not responding to therapy, both stable disease and partial response was classified as responding to therapy. These conditions classified as

responding to therapy, were also confirmed to be statistically different to the non-treated vehicle controls, using methods described below.

Statistical analysis

All experimental groups were performed in technical triplicates. Results are presented as mean ± standard deviation. Statistical differences in treatment responses were analyzed using a repeated measurements analysis of variance (2-way ANOVA) for multiple hypotheses using Dunnett's multi comparison post hoc test. Data analysis was performed using Prism GraphPad Software. In all tests, p values < 0.05 were considered statistically significant.

The validity of the *in vitro* treatment response (IVTR), and the dosages and methods used to assess these responses, was tested by comparing each IVTR outcomes set to the corresponding strict clinical treatment responses (SCTR) or extended clinical treatment responses (ECTR). Diagnostic agreement was assessed using unweighted kappa values, positive predictive values (PPV), negative predictive values (NPV), sensitivity and specificity. Sensitivity and specificity are generally used for comparing a new test against a test that is currently the golden standard. However in this study, we aim to predict the change that a patient does or does not respond to an administered therapy, based on the positive or negative results of an *in vitro* test. This can be determined using the predictive values of the obtained test result. These predictive values indicate the ability of the obtained IVTR to confirm the presence or absence of a clinical treatment response, its outcome ranging from +1 to 0, where 0.5 represents the prediction of a random chance, and 1 represents a perfect prediction.[5] Kappa values (or Cohen's kappa [6]) are widely used to measure the degree of agreement between two observers, or in our case the degree of agreement between *in vitro* and clinical treatment outcomes. Its outcome can range from −1 to +1, where 0 represents the agreement of a random chance, and 1 represents perfect agreement between the compared data sets. The interpretation of kappa values is subject to some debate, but in general interpreted as follows: < 0.20 = poor, 0.21 - 0.40 = fair, 0.41 - 0.60 = moderate, 0.61 - 0.80 = good, and 0.81 - 1.00 is very good.[7]

References

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Supplementary Tables

Supplementary Table 1 Diagnostic agreement between the *in vitro* treatment responses (IVTR) compared to the corresponding strict clinical treatment responses (SCTR) or extended clinical treatment responses (ECTR) of all treatment. CTR= clinical treatment response, N = sample size, TN = true negative, FN = false negative, FP = false positive, TP = true positive, Sen = Sensitivity, Spe = Specificity, PPV = positive predictive value, NPV = negative predictive values, Kappa = unweighted kappa value.

All treatments												
IVTR		CTR	N	TN	FN	FP	TP	Sen	Spe	PPV	NPV	Kappa
Analysis method	Dose											
% dead myeloma cells	1 x	SCTR	14	6	3	1	4	0.571	0.857	0.800	0.667	0.429
% dead myeloma cells	2 x	SCTR	14	2	2	4	6	0.750	0.333	0.600	0.500	0.087
% dead myeloma cells	1 x	ECTR	26	7	10	1	8	0.444	0.875	0.889	0.412	0.243
% dead myeloma cells	2 x	ECTR	26	3	6	4	13	0.684	0.429	0.765	0.333	0.103
# living myeloma cells	1 x	SCTR	14	2	5	5	2	0.286	0.286	0.286	0.286	-0.429
# living myeloma cells	2 x	SCTR	14	1	2	6	5	0.714	0.143	0.455	0.333	-0.143
# living myeloma cells	1 x	ECTR	26	3	8	5	10	0.556	0.375	0.667	0.273	-0.063
# living myeloma cells	2 x	ECTR	26	2	5	6	13	0.722	0.250	0.684	0.286	-0.029

Supplementary Table 2 Diagnostic agreement between the *in vitro* treatment responses (IVTR) compared to the corresponding strict clinical treatment responses (SCTR) or extended clinical treatment responses (ECTR) of alkylating agents and proteasome inhibitors. CTR= clinical treatment response, N = sample size, TN = true negative, FN = false negative, FP = false positive, TP = true positive, Sen = Sensitivity, Spe = Specificity, PPV = positive predictive value, NPV = negative predictive values, Kappa = unweighted kappa value.

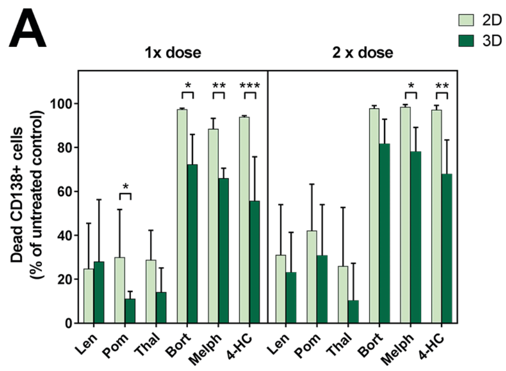
Alkylating agents and proteasome inhibitors												
IVTR		CTR	N	TN	FN	FP	TP	Sen	Spe	PPV	NPV	Kappa
Analysis method	Dose											
% dead myeloma cells	1 x	SCTR	8	4	1	0	3	0.750	1.000	1.000	0.800	0.750
% dead myeloma cells	2 x	SCTR	8	3	0	1	4	1.000	0.750	0.800	1.000	0.750
% dead myeloma cells	1 x	ECTR	16	4	5	0	7	0.583	1.000	1.000	0.444	0.412
% dead myeloma cells	2 x	ECTR	16	3	2	1	10	0.833	0.750	0.909	0.600	0.538
# living myeloma cells	1 x	SCTR	8	0	2	4	2	0.500	0.000	0.333	0.000	-0.500
# living myeloma cells	2 x	SCTR	8	1	1	3	3	0.750	0.250	0.500	0.500	0.000
# living myeloma cells	1 x	ECTR	16	0	3	4	9	0.750	0.000	0.692	0.000	-0.273
# living myeloma cells	2 x	ECTR	16	1	3	3	9	0.750	0.250	0.750	0.250	0.000

Supplementary Table 3 Diagnostic agreement between the *in vitro* treatment responses (IVTR) compared to the corresponding strict clinical treatment responses (SCTR) or extended clinical treatment responses (ECTR) of immunomodulatory drugs. CTR= clinical treatment response, N = sample size, TN = true negative, FN = false negative, FP = false positive, TP = true positive, Sen = Sensitivity, Spe = Specificity, PPV = positive predictive value, NPV = negative predictive values, Kappa = unweighted kappa value.

Immunomodulatory drugs												
IVTR		CTR	N	TN	FN	FP	TP	Sen	Spe	PPV	NPV	Kappa
Analysis method	Dose											
% dead myeloma cells	1 x	SCTR	6	2	2	1	1	0.333	0.667	0.500	0.500	0.000
% dead myeloma cells	2 x	SCTR	6	0	1	3	2	0.667	0.000	0.400	0.000	-0.333
% dead myeloma cells	1 x	ECTR	10	3	5	1	1	0.167	0.750	0.500	0.375	-0.071
% dead myeloma cells	2 x	ECTR	10	1	3	3	3	0.500	0.250	0.500	0.250	-0.250
# living myeloma cells	1 x	SCTR	6	2	3	1	0	0.000	0.667	0.000	0.400	-0.333
# living myeloma cells	2 x	SCTR	6	0	1	3	2	0.667	0.000	0.400	0.000	-0.333
# living myeloma cells	1 x	ECTR	10	3	5	1	1	0.167	0.750	0.500	0.375	-0.071
# living myeloma cells	2 x	ECTR	10	1	2	3	4	0.667	0.250	0.571	0.333	-0.087

Legend to Supplementary Figure

Supplementary Figure 1 Therapy responses of myeloma cell lines, comparing 2D vs 3D cultures, and flow cytometry vs confocal analysis. (A) Flow cytometric analysis of myeloma cell lines (OPM2 and L363) cultured in 2D, or in 3D with supporting MSCs and EPCs, 72 hours after treatment addition. Myeloma cells cultured in 3D were less sensitive to the given therapies, in both dosages tested. **(B)** The percentage of dead myeloma cells after treatment. The myeloma cell lines (OPM2 and L363) cultured in 3D after 72 hours, quantified using either flow cytometry or confocal imaging. **(C)** Confocal image of L363 co-cultured with MSCs and EPCs, and treated with 4 µM lenalidomide for 72 hours. Live cells are shown in green (calcein), dead cells are shown in red (ethidium homodimer-1). L363 cells can be morphologically identified by their round, clustered outgrowth (red arrows). Supporting MSCs and EPCs can morphologically identified as elongated cells, forming cell-cell networks (yellow arrows). Within one culture, different regional survival of cells can be observed. Data is presented as mean ± SD. Abbreviations: len = lenalidomide, pom = pomalidomide, thal = thalidomide, bort = bortezomib, carf = carfilzomib, melp = melphalan, 4-HC = 4-hydroperoxy-cyclophosphamide.


A
1x dose
2 x dose
2D
3D
Dead CD138+ cells
(% of untreated control)
0
20
40
60
80
100
*
*
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***
*
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Len
Pom
Thal
Bort
Melph
4-HC
Len
Pom
Thal
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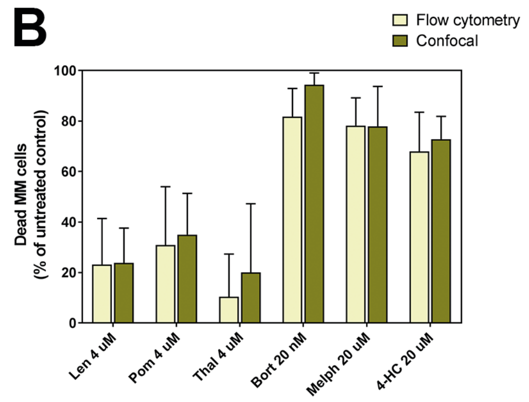
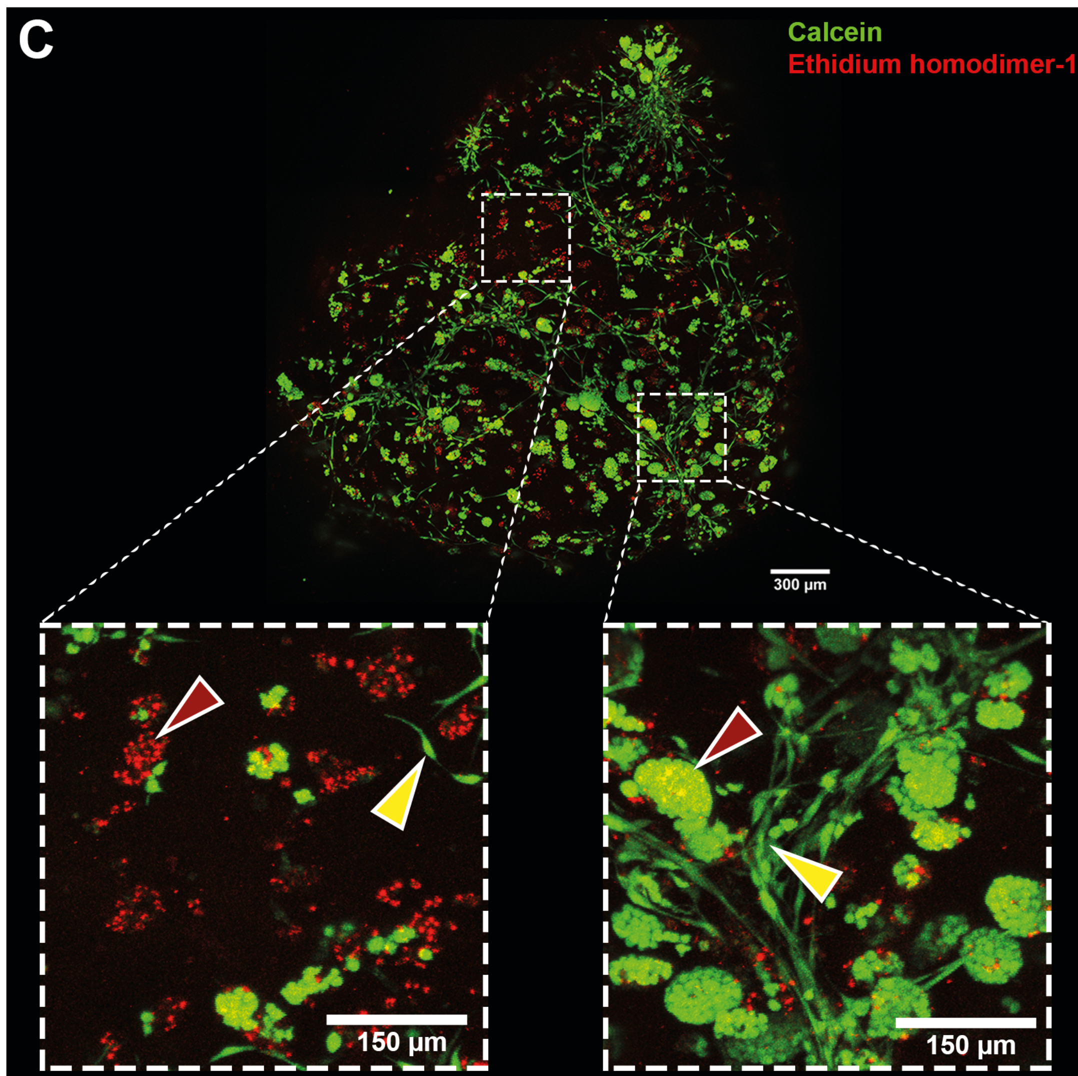

B
Flow cytometry
Confocal
Dead MM cells
(% of untreated control)
0
20
40
60
80
100
Len 4 uM
Pom 4 uM
Thal 4 uM
Bort 20 nM
Melph 20 uM
4-HC 20 uM


C
Calcein
Ethidium homodimer-1
300 μm
150 μm
150 μm