SUPPLEMENTARY APPENDIX

Activity of everolimus (RAD001) in relapsed and/or refractory multiple myeloma: a phase I study

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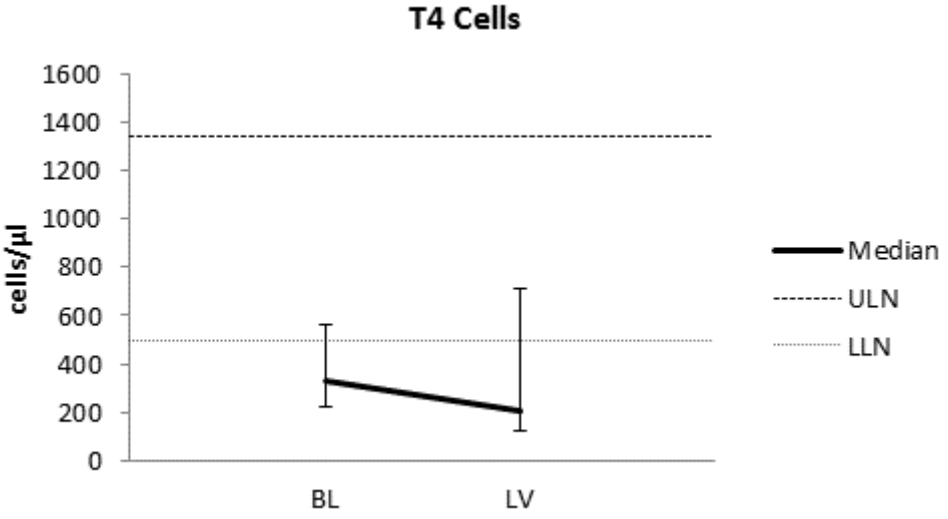
SUPPLEMENTAL DATA

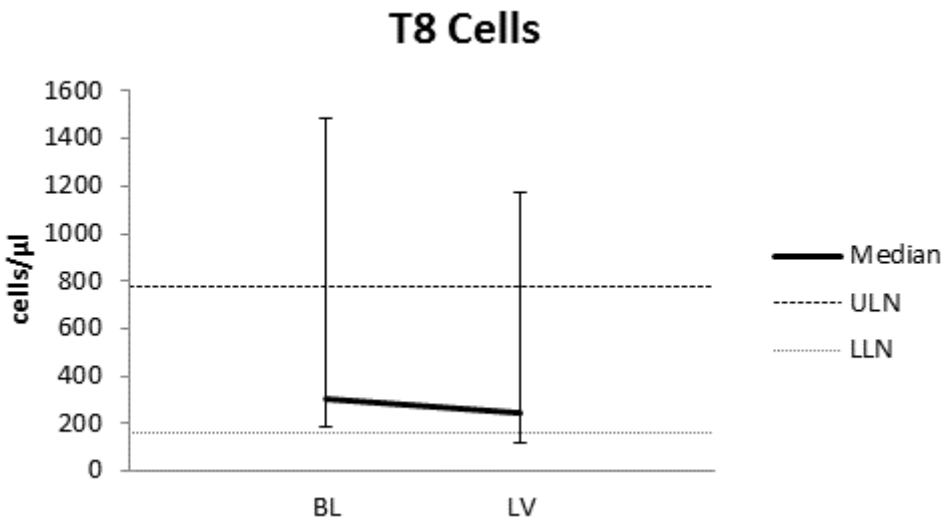
Pharmacokinetics: Everolimus serum levels

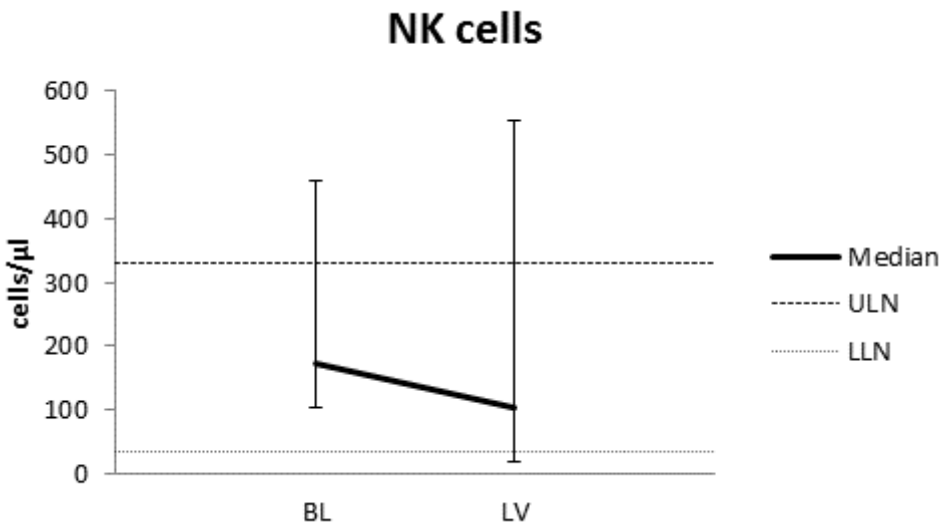
DOSE	INTENDED EVEROLIMUS BLOOD LEVEL (ng/ml)	ACHIEVED EVEROLIMUS BLOOD LEVEL (ng/ml)			
		Day 8		Day 28	
		RANGE	MEDIAN	RANGE	MEDIAN
5 mg	3.0-9.0	2.5-14.9	4.4	3.6-12.7	7.25
7.5 mg	4.8-14.3	4.0-23.0	19.7	4.6-23.2	8.0
10 mg	6.5-19.0	2.5-12	6.7	3.0-15.1	5.2

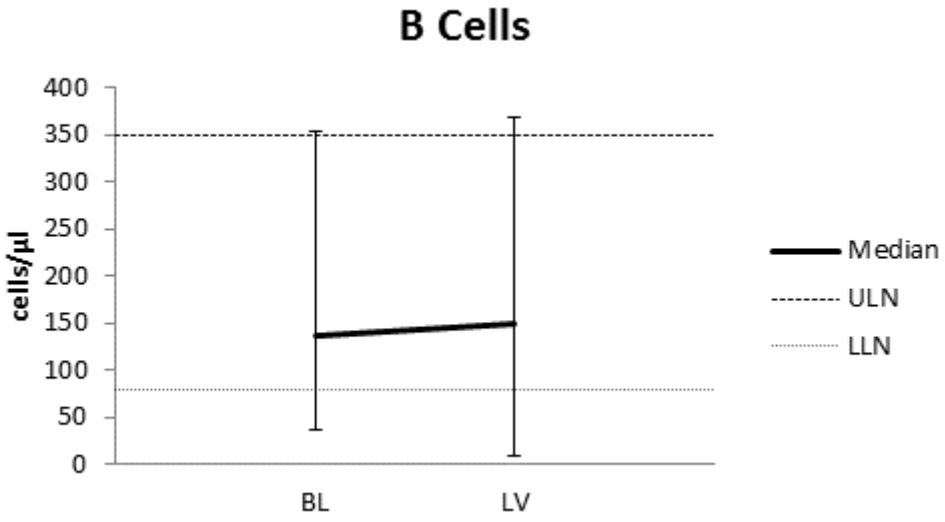
Effect on the lymphocyte subset levels

Monitoring of lymphocyte subsets was performed at several times during treatment. Most patients showed reduced T4 cell counts at baseline while T8, B cells and NK cells were generally normal. However, no significant changes were induced by the treatment (paired two-tailed T-test: T4 cells: p=0.36, T8 cells: p=0.10, NK cells: p=0.64, B cells: p=0.76). The figures compare the median value at baseline (BL) with the last available value (LV) of all patients. Normal ranges (ULN= upper limit of normal; LLN= lower limit of normal) are indicated by dotted lines; the bars show minimal/maximal values.






NK cells
600
500
400
300
200
100
0
cells/µl
BL
LV
Median
ULN
LLN


B Cells
400
350
300
250
200
150
100
50
0
cells/µl
BL
LV
Median
ULN
LLN

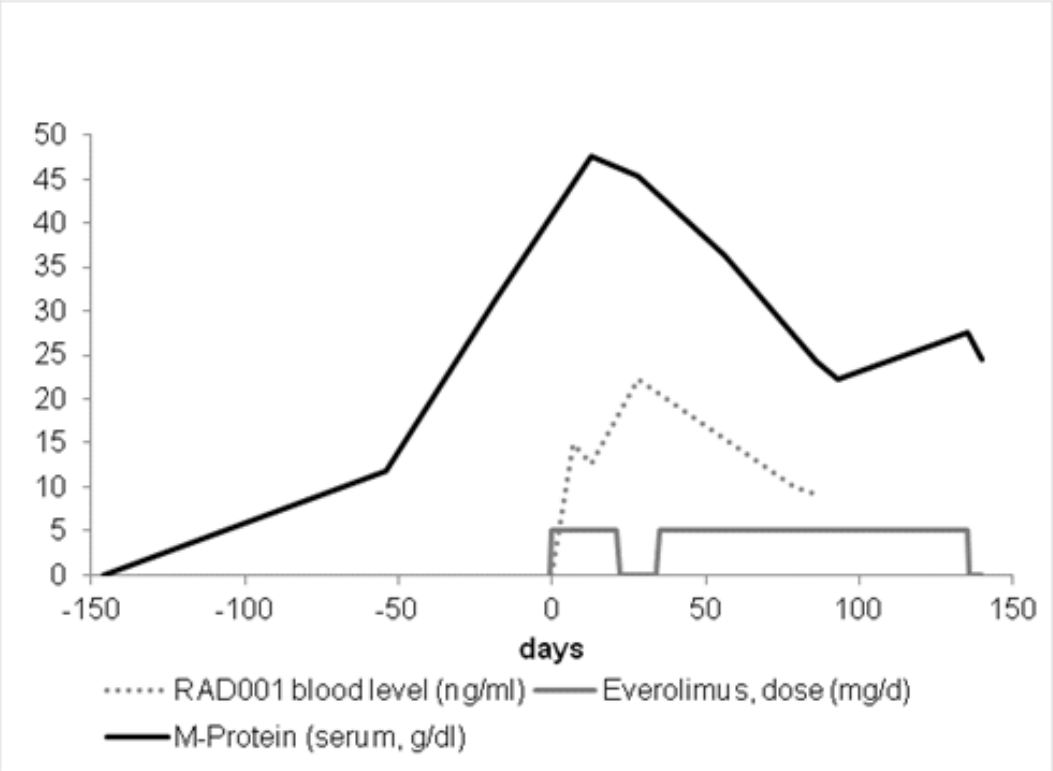
Clinical Benefit/Response

PATIENT	DOSE	DURATION OF TREATMENT (days)	RESPONSE/DISEASE STATUS		REASON FOR DISCONTINUATION
			best	6 cycles	
001-001	5 mg	117	SD (MR)	n.a.	PD
001-002	5 (10) mg	90	SD	n.a.	PD
001-004	5 mg	41	PD	n.a.	PD
001-005	5 mg	135	PR	n.a.	PD *
001-006	7,5 mg	278	SD	SD	PD after end of study (> 6 months)
001-007	7.5 mg	105	SD	n.a.	PD
001-008	7.5 mg	33	PD	n.a.	PD [§]
001-009	10 mg	7	n.a.	n.a.	Withdraw of consent
001-010	10 mg	82	SD	n.a.	PD
001-011	10 mg	180	SD	SD	End of study
001-012	10 mg	42	PD	n.a.	PD
001-013	10 mg	96	SD	n.a.	PD [#]
001-014	10 mg	246	SD	SD	PD after end of study (> 6 months)
003-001	10 mg	13	PD	n.a.	PD**
003-002	10mg	14	PD	n.a.	PD**
003-003	10 mg	39	SD	n.a.	Infection / PD

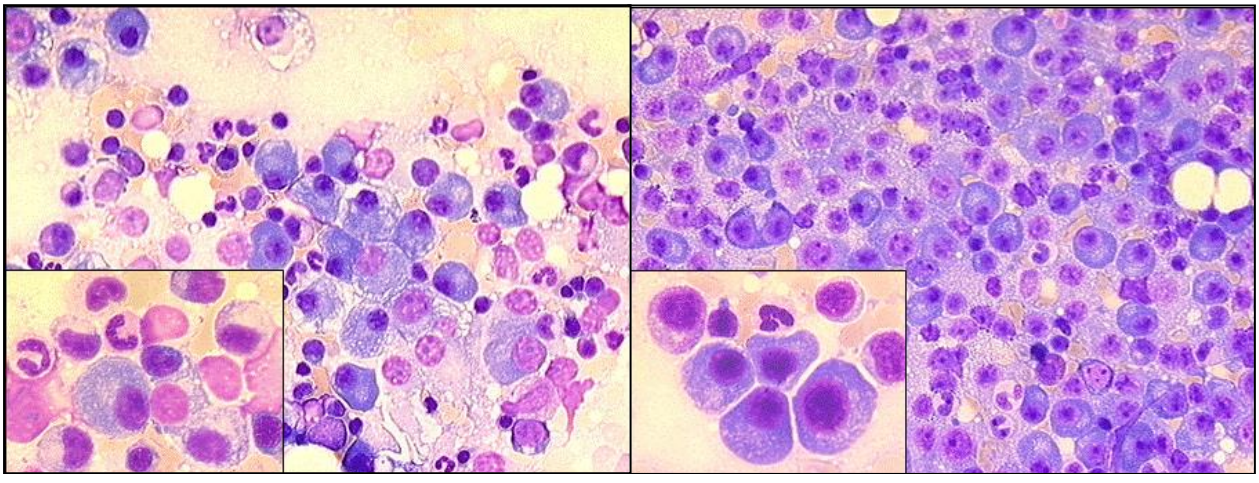
PD = progressive disease, SD = stable disease, PR = partial remission, MR= minor response; *isolated progression of a sternal tumor, ** early withdraw due to hyper viscosity, [§] spinal tumor, [#] pleural effusion, n.a. = not applicable

Partial response in a heavily pretreated patient

One very heavily pretreated patient achieved partial response in the first cohort before isolated progression of a sternal tumor was observed. The figure shows serum M protein levels, the dose of everolimus and the achieved serum levels of everolimus.

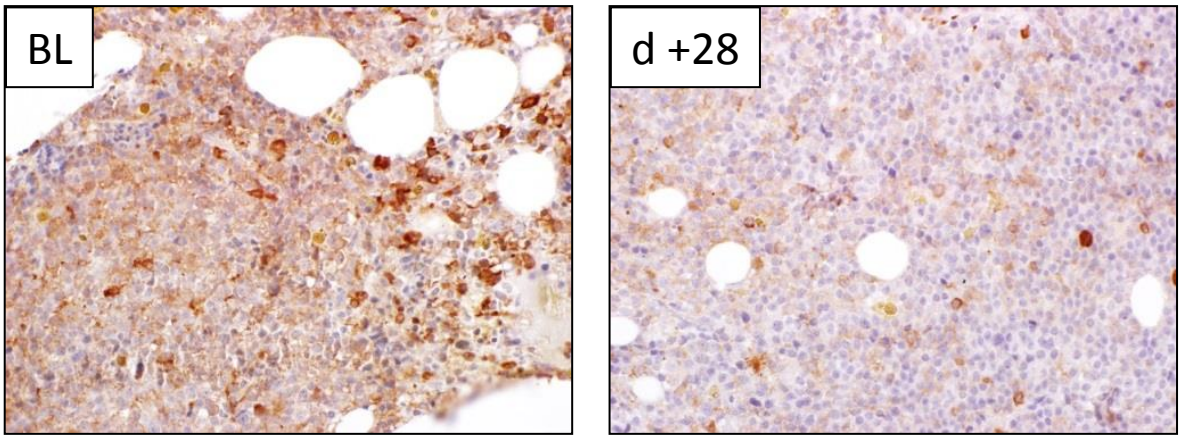


Reduction of malignant plasma cells in the patient with PR could be confirmed by bone marrow aspirate. Whereas screening smear (left) showed subtotal plasma cell infiltration, at the end of study recovery of normal bone marrow cells could be observed with a residual plasma cell infiltration of approx. 30 to 40 % (right). Large figure shows 400-fold magnification; a more detailed view is in the left lower corner (1000-fold).



Bone Marrow: Imunohistology, phopho S6

Bone marrow biopsies were taken at screening and at day 28 of treatment to elucidate the everolimus activity in the bone marrow . Paraffin-embedded sections were stained for phosphorylated S6 protein as a downstream target of mToR. The screening biopsy of patient 001-008 showed high activity of the pathway in bystander cells as well as in malignant plasma cells (above: x 400 magnifications). Upon 28 days of treatment with 7.5 mg everolimus daily S6 phosphorylation was generally reduced but not totally abolished (below).



METHODS – DETAILED VERSION

Study design

The trial CRAD001C2455 was designed as an open-label, multi-center phase I trial of continuous, escalating doses of everolimus once daily in patients age ≥ 18 years who were relapsed or refractory for MM after two prior treatment lines. The primary objective was to determine the maximum tolerated dose (MTD) of everolimus and dose-limiting toxicities (DLTs). Secondary objectives included safety and tolerability, clinical activity of everolimus and efficacy per IMWG criteria. At least 3 patients were included at each dose level until MTD or the highest dose level was reached, at which a total of 6 patients should be treated.

The evaluable patient population for the determination of MTD consisted of patients who have been treated for at least 28 days or suffered DLT. Patients, who did not meet these minimum study evaluations, were regarded as ineligible for the MTD-determining population and were replaced. The treatment was planned for six 28 day cycles if no progressive disease or DLT was observed. However, an additional treatment with study drug was allowed for patients achieving at least stable disease after six cycles until disease progression or limiting toxicity.

The project management and trial monitoring was performed by the clinical research organization CROLL, Nürnberg, Germany, and the data analysis was centrally performed by the Estimate GmbH, Augsburg, Germany.

Patients

Adult patients with relapsed or refractory MM of Salmon and Durie stage ≥ II after failure of at least two prior treatment regimens were enrolled. The study was designed in accordance with the International Conference on Harmonisation (ICH)

Harmonised Tripartite Guidelines for Good Clinical Practice, with applicable local regulations and the ethical principles of the Declaration of Helsinki. The protocol was approved by the Institutional Review Board/Independent Ethics Committee/Research Ethics Board at each study site, and informed consent was obtained from all patients (EudraCT number: 2006-002675-41).

Key inclusion criteria were WHO performance status ≤ 2, measurable disease marker according to the "international uniform response criteria" [1], adequate bone marrow function as shown by leukocytes > 2,500/mm^3, platelets >50,000/mm^3 and hemoglobin ≥ 8g/dl, life expectancy > 6 months, and adequate liver function as shown by serum bilirubin ≤ 1.5 x upper limit of normal (ULN) and serum transaminases activity ≤ 3 x ULN. Patients with other concurrent severe and/or uncontrolled medical disease, which could compromise participation in the study, or previous treatment with mToR inhibitors were excluded.

Safety and efficacy assessments

Patients were monitored for safety throughout the trial and up to 28 days following the last dose of study treatment. Patients were assessed for efficacy at baseline, at the beginning of each cycle, at the end of treatment and whenever clinically indicated. Response status was based on investigator assessment using IMWG criteria. Assessment of minimal response (MR) as per the International Myeloma Workshop Consensus Panel I was evaluated as an exploratory endpoint.

Pharmacokinetic analyses and drug adjustment

The everolimus concentrations in whole blood were determined by a validated liquid chromatography method with mass spectrometry (LC-MS) following liquid/liquid extraction (Central Laboratory of the University Hospital of Göttingen, Germany). The

blood dose level assessment was performed after 8 and 28 days (or more often when the intended dose level was not achieved). In cohort 1 of the dose escalation phase, the starting dose was 5 mg everolimus, followed by cohorts with 7.5 mg and 10 mg. The intended blood level in the particular cohorts was as follows: 6 ng/ml for cohort 1 (5 mg daily), 9.5 ng/ml for cohort 2 (7.5 mg daily) and 13 ng/ml for cohort 3 (10 mg daily). If a patient showed a significant deviation from the intended blood drug level (defined as +/- 50%) not manageable by changes of concomitant medication or other factors, dose had to be adjusted, however not exceeding the dose of 10 mg daily. In case of dose adjustments the patient had to be replaced per protocol. To investigate the correlation of the median achieved drug level and the M protein response, the Pearson product-moment correlation coefficient was calculated (2-tailed significance, SPSS 13).

Bone marrow assessment

Bone marrow aspirates and biopsies were performed during screening, 28 days upon treatment, at the end of study and when clinically indicated. The aspirates were stained by Giemsa and the biopsies with hematoxylin and eosin for morphological assessment. Immunohistochemical stainings for mToR (total and phosphorylated) for the downstream targets 4E-binding protein 1 (4EBP1, total and phosphorylated) and phosphorylated S6 ribosomal protein (S6) were performed using antibodies from Cell Signaling (Danvers, USA), as described previously [2]. In a few patients bone marrow investigation could not be performed as intended, since the biopsies were not representative (extra-medullary disease, previous irradiation or technical reasons) or due to patient denial.

Effect on the immune system

To elucidate the biological effect of everolimus on the immune system of MM patients in more detail the level of the not involved immunoglobulin classes were determined by turbidimetric measurement (Cobas c systeme, Roche Diagnostics, Mannheim, Germany) and lymphocyte subsets were assessed by FACS staining as described previously (antibodies provided by Beckman Coulter, Krefeld, Germany) [3]. The values assessed at baseline were compared to the last value achieved during treatment and tested for significant changes using of the paired two-tailed T-test (SPSS 13), In two patients under treatment cytotoxic activities of natural killer (NK) cells were determined in a standard 4 h ^{51}Cr release assays performed as described previously [4]. Briefly, NK cells were isolated from peripheral blood by negative selection using magnetic activated cell sorting (Miltenyi Biotech, Bergisch Gladbach, Germany) and applied as effector cells against the NK cell sensitive cell line K562 at varying effector-to-target cell (E:T) ratios. ^{51}Cr release from triplicates was determined in counts per minute (cpm). Results were compared to a healthy volunteer.

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