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Supporting Online Material for

Tumor Regression in Cancer Patients by Very Low Doses of a T Cell–Engaging Antibody

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SUPPLEMENTARY ONLINE MATERIALS

Tumor Regression in Cancer Patients by Very Low Doses of a T Cell-engaging Antibody

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MATERIALS AND METHODS

Patient treatment and monitoring

According to the study protocol, patients with relapsed histologically confirmed, indolent B-cell non-Hodgkin's lymphoma (B-NHL) including mantle cell lymphoma were recruited in an ongoing open-label, multi-center phase I interpatient dose-escalation trial. The majority of the 39 patients thus far enrolled in this Phase 1 dose escalation study had been diagnosed with mantle cell lymphoma (MCL; 38.5%) and follicular lymphoma (FL; 41%), and previously treated with multiple courses of diverse chemotherapeutics (median of 3 regimens) and anti-CD20 antibody rituximab (87% of patients).

Measurable disease (at least one lesion $\geq$ 1.5 cm) as documented by CT scan was required for inclusion into the study. Patients received blinatumomab by continuous intravenous infusion with a portable minipump system over 4-8 weeks at different flow rates (i.e., dose levels). This led to sustained and predictable serum levels of the BiTE antibody over the entire treatment period. Methyl-prednisolone and low molecular weight heparin were prophylactically given for the first treatment days in order to curb potential side effects related to first infusion reactions. Patients were hospitalized during the first two weeks of treatment for close monitoring of safety events in this phase 1 trial, Subsequently, they were released from the hospital and continued treatment at home. Patients without evidence of disease progression after four weeks were offered to continue treatment for additional 4 weeks and/or

one or two additional treatment cycles. The protocol did not foresee to re-treat patients at higher dose levels.

So far, six different dose levels have been completed: 0.0005, 0.0015, 0.005, 0.015, 0.03 and 0.06 mg/m^2/24 h. Cohorts consisted of three patients each, if no adverse events defined by the study protocol as DLT (dose limiting toxicity) were observed. The DLT period was 2 weeks. In case of one DLT among the first three patients the cohort was expanded to six patients, which – in the absence of a second DLT – allowed further dose escalation (3+3 design), as decided by a data review committee (DRC). Accordingly, cohorts with three patients without DLT and cohorts with six patients with one DLT were defined as a safety profile that warranted further escalation. Study treatment was stopped in all patients who developed a DLT. At 0.0015 mg/m^2/24 h different steroid doses were tested in two cohorts of three patients each. At 0.015 and 0.03 mg/m^2/24 h, different modes of treatment initiation during the first 24 h were tested in additional cohorts. The cohort of 0.06 mg/m^2 per day was expanded in order to test two different clinical trial materials.

Assessment of efficacy as based on scanning of patients by computer tomography (CT) was performed by central reference radiology after 4 weeks of treatment. Patients receiving additional 4-week cycles of treatment were examined by additional CT scans directly following or four weeks after end of treatment. Disappearance or normalization in size of all known lesions (including an enlarged spleen) plus clearance of bone marrow from lymphoma cells in cases of bone marrow infiltration was counted as complete response (CR). Reduction by at least 50% from baseline of the sum of products of the two longest diameters (SPD) of each predefined target lesion was defined as partial response (PR); a reduction by at least 25% was regarded a minimal response (MR). Progressive disease (PD) was defined as ≥50% increase of SPD from baseline. SPD deviations from baseline between +50% and –25% were regarded as stable disease (SD). The study protocol was approved by the independent

ethics committees of all participating centers and sent for notification to the responsible regulatory authority.

Four-color FACS analysis of lymphocyte subpopulations

Collection of blood samples and routine analysis

Patient blood samples (6 ml) were obtained before and 0.75, 2, 6, 12, 24, 30, 48 hours after start of blinatumomab infusion as well as on treatment days 8, 15, 17, 22, 24, 29, 36, 43, 50, 57 and 4 weeks after end of blinatumomab infusion using EDTA-containing Vacutainer™ tubes (Becton Dickinson), which were shipped for analysis at 4°C. In some cases slight variations of these time points occurred for operational reasons. FACS analysis of lymphocyte subpopulations was performed within 24–48 h after blood sample collection. Absolute numbers of leukocyte subpopulations in the blood samples were determined through differential blood analysis on a CoulterCounter™ (Coulter).

Isolation of PBMC from blood samples

Peripheral blood mononuclear cells (PBMC) isolation was performed by an adapted Ficoll™ gradient separation protocol. Blood was transferred at room temperature into 10 ml Leucosep™ tubes (Greiner) pre-loaded with 3 ml Biocoll™ solution (Biochrom). Centrifugation was carried out in a swing-out rotor for 15 min at 1,700x g and 22°C without deceleration. PBMC on top of the Biocoll™ layer were isolated, washed once with FACS buffer (PBS/2%; fetal bovine serum [Biochrom]), centrifuged and resuspended in FACS buffer. Centrifugation during all wash steps was carried out in a swing-out rotor for 4 min at 800x g and 4°C. If necessary, lysis of erythrocytes was performed by incubating the isolated PBMC in 3 ml erythrocyte lysis buffer (8.29 g NH_4Cl, 1.00 g $KHCO_3$, 0.037 g EDTA, ad 1.0 l H_2O_{bidest}, pH 7.5) for 5 min at room temperature followed by a washing step with FACS buffer.

Staining of PBMC with fluorescence-labelled antibodies against cell surface molecules

Monoclonal antibodies were obtained from Invitrogen, Dako or Becton Dickinson and used according to the manufacturers` recommendations. For staining, $5x10^5$–$1x10^6$ cells were used for antibody combinations each consisting of 4 different antibodies linked to different fluorescence dyes (see table below). Cells were pelleted in V-shaped 96-well multititer plates (Greiner) and the supernatant was removed. Cell pellets were resuspended in a total volume of 100 µl containing the specific antibodies diluted in FACS buffer. Incubation was carried out in the dark for 30 min at 4°C. Subsequently, samples were washed twice with FACS buffer and cell pellets resuspended in FACS buffer for flowcytometric analysis.

Flowcytometric detection of stained lymphocytes by FACS

Data collection was performed with a 4-color BD FACSCalibur™ instrument (Becton Dickinson). For each antibody combination, $1x10^4$ cells of defined lymphocyte subpopulations were acquired. Statistical analysis was performed with the program CellQuest Pro™ (Becton Dickinson) to obtain lymphocyte subpopulation percentages and to classify cell surface molecule expression intensity. Subsequently, percentages were correlated with the lymphocyte count from the differential blood analysis to calculate absolute cell numbers of lymphocyte subpopulations. T cell activation was analysed as percentage of $CD8^+$ or $CD4^+$ T cells positive for a specific activation marker (CD69, CD25 or HLA-DR). B cell apoptosis was measured as mean fluorescence intensity (MFI) of the annexin V staining of B cells.

Antibody Combinations Used

No.	FITC	PE	PerCP	APC
1	Anti-CD13[#] / Anti-CD14[#]	Anti-CD56	Anti-CD3	Anti-CD19[§]
2	Anti-gamma/delta TCR[#]	Anti-CD8	Anti-CD4	Anti-CD3
3	Anti-CD45RA	Anti-CD197	Anti-CD8	Anti-CD28
4	Anti-HLA-DR	Anti-CD69	Anti-CD8	Anti-CD25
5	Anti-CD45RA	Annexin V	Anti-CD20	Anti-CD28

Sources: Becton Dickinson, [#]Invitrogen, [§]Dako

Quantification of blinatumomab in serum or plasma

Quantification of blinatumomab in human serum and plasma was performed using a combined method composed of a cytotoxicity assay with subsequent determination of CD69 activation on the surface of T cells via flow cytometry. To this end, Raji lymphoma line as target and human T cell line HPBALL as effector cells were incubated in the presence of calibration standard serial dilutions of blinatumomab in serum or plasma ranging from 200 ng to 3 pg/ml. A blank value is used to measure background response of the assay. Serum or plasma samples from patients were pre-diluted to ensure measurement in the linear range of the assay.

Either working standard, blank or patient sample was added in the presence of 50% human serum to target and effector cells. After incubation of the cell suspension for 14-18 hours at 37°C +/- 2°C in a CO_2 incubator, cells were labeled with a fluorescent antibody directed against T cell activation surface marker CD69 and measured in a FACS Calibur instrument. HPBALL T cells expressing CD69 were detected and quantitated using forward and sideward scatter. Cell Quest software was used to determine mean fluorescence intensity values. Quality control samples of low, medium and high blinatumomab concentration and

unknown blinatumomab concentrations in serum or plasma were determined using a blinatumomab calibration standard curve applying the software program GraphPadPrism.

Determination of cytokines in patient samples

The cytokine levels in patient serum were determined with the BD^{TM} CBA Human Th1/Th2 Cytokine Kit II (Becton Dickinson). This FACS-based assay quantitatively measures the in a single serum sample the levelsof IL-2, IL-4, IL-6, IL-10, TNF-alpha and IFN-gamma. Six bead populations with distinct FL3 fluorescence intensities have been coated with capture antibodies specific for these six cytokines. The six bead populations are mixed and then incubated with the PE-conjugated detection antibodies and the test samples or the standards to form sandwich complexes. Following acquisition of sample data using flow cytometery, the sample results are generated in graphical and tabular format using the CBA Analysis Software (Becton Dickinson). The method is validated and detailed in the instruction manual of the kit.

Immunohistochemistry (IHC)

Immunohistochemical staining was performed on formalin-fixed paraffin-embedded tissue according to an established procedure (H.U. Völker *et al., Diagn Pathol*. **2**, 1 (2007)). The following monoclonal antibodies were used at dilutions indicated: anti-CD3 (1:80, clone: PS1, Novocastra), anti-CD5 (1:600; clone: 4C7, Novocastra), anti-CD20 (1:500, clone: L26, Dako), anti-cyclin D1 (1:80; clone: 1DGM, Novocastra).

SUPPLEMENTARY DATA

Description of all clinical events that led to a discontinuation of treatment with blinatumomab

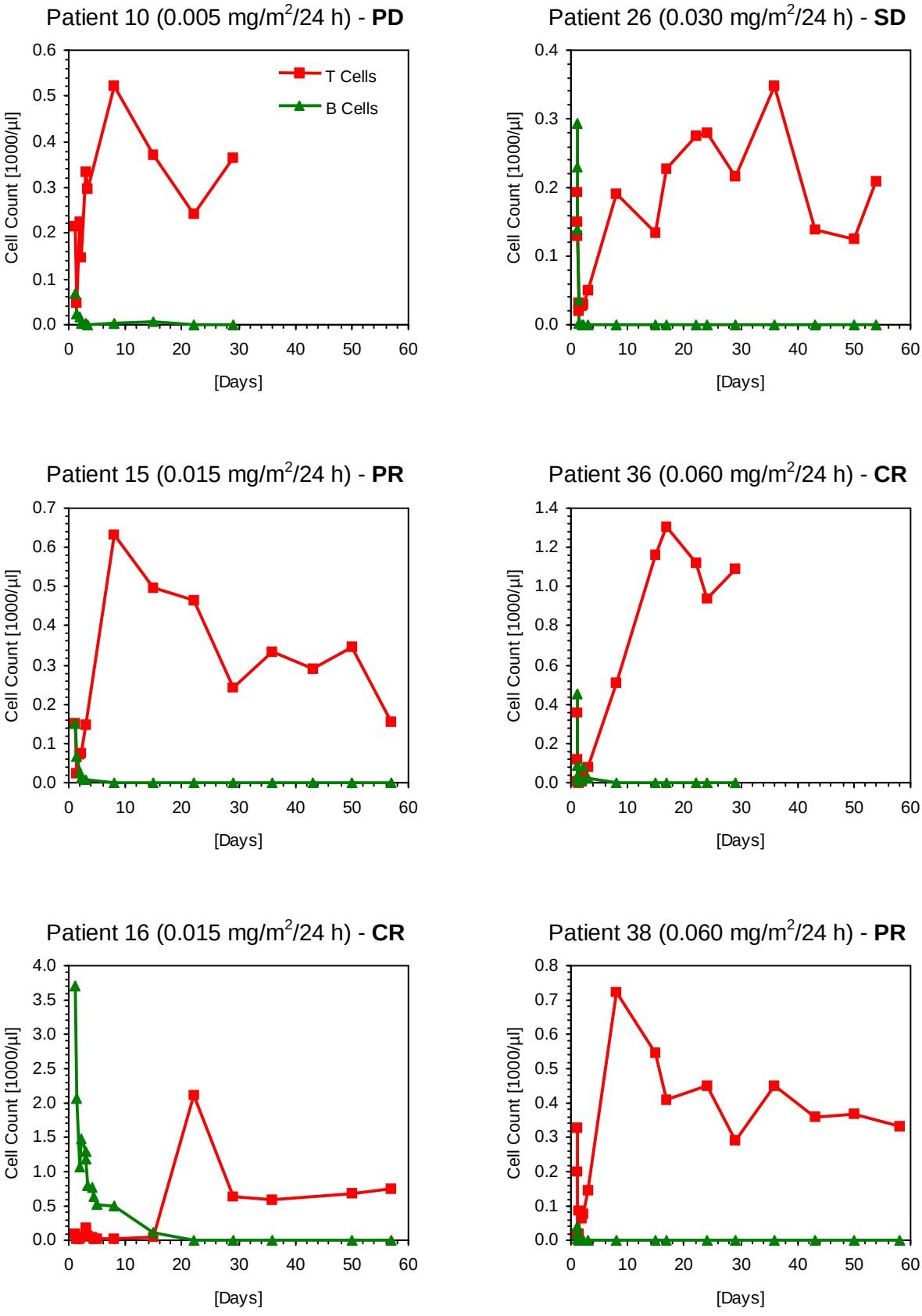
One patient treated at 0.015 mg/m^2 per day developed a sepsis after 39 days leading to discontinuation of treatment on the same day. The patient with known pre-existing episodes of nearly fatal sepsis succumbed to the sepsis one day later. Another patient at this dose level with a pre-existing hypogammaglobulinaemia and a medical history of intermittent infections was discontinued after 3 days because of pneumonia. One patient at dose level of 0.03 mg/m^2 per day with pre-existing episodes of acidosis caused by a renal insufficiency was discontinued after 2 days of treatment with blinatumomab due to the development of a reversible metabolic acidosis (grade 4), accompanied by a generalized seizure. In five patients, treatment with blinatumomab was discontinued after development of CNS-related events. Two patients had confusion (grade 2, at a dose level of 0.015 mg/ m^2 per day) during the first 48 hours after treatment initiation, and two patients presented with cerebellar symptoms, such as kinetic tremor and ataxia (grades 2 and 3, at dose level of 0.06 mg/ m^2 per day), which first appeared in the second and fourth week of treatment with blinatumomab, respectively. All CNS-related events leading to discontinuation of treatment were fully reversible within 1-3 days after discontinuation of treatment without sequelae. Despite treatment discontinuation (see Tab. S1), both patients who developed cerebellar symptoms had an objective clinical benefit (1 PR and 1 CR). Treatment of a first patient at a dose level of 0.09 mg/m^2 per day was discontinued after 2 days because of seizures, which did not recur after discontinuation of treatment. This patient also fully recovered without sequelae. In violation of the study protocol, the patient presented at the time of treatment start with an acute airway infection treated by intravenous administration of the antibiotic cefuroxime. The patient was also on theophylline therapy for chronic obstructive lung disease. Shortly before

treatment start, C-reactive protein values were increasing, and the percentage of pre-activated HLA-DR-positive CD8+ T cells was highest of all patients in the study (i.e., 83%).

Cytokine responses in patients

TNF-α was transiently increased over baseline in only 6 patients with serum peak levels in the range of 125 and 450 pg/ml in 5 patients, and at 1,420 pg/ml in one patient. An initial transient elevation of the anti-inflammatory cytokine IL-10 was observed in 25 out of 39 patients, of whom 19 also showed a transient elevation of pro-inflammatory cytokines IL-6 or IFN-γ. Four patients showed an isolated peak of IL-6 or IFN-γ.

SUPPLEMENTARY FIGURE S1



Legend for Supplementary Figure

Figure S1. Kinetics for peripheral T and B cell counts of six more patients treated with blinatumomab. Treatment details for each patient are given in Table S1. Patients were selected to compare different dose levels and treatment outcomes. Cell counts were determined by fluorescence-activated cell sorting. Red: counts of peripheral T cells determined by anti-CD3 antibody staining; green: counts of peripheral B cells determined by anti-CD19 antibody staining. Abbreviations used are: CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

SUPPLEMENTARY TABLES

Table S1

Cohort	Patient	Age/ Sex	Stage of Disease - Number of prior systemic therapies	Dose Level [mg/m^2/Day]	Period Treated [Days]	Total Dose [mg]	Sustained Peripheral B Cell Depletion	Clearance of Bone Marrow	Best Response* (Duration in Months; + = ongoing)
1	1	71/m	IC, Binet C - 6	0.0005	28	0.025	-[b]	None	SD
	2	67/f	MCL, Stage IV/A/E - 2	0.0005	28	0.025	-	n.d.	PD
	3	67/m	CLL, Stage IV/B/E - 4	0.0005	56	0.05	-	n.d.	MR
2[a]	4	69/m	MCL, Stage IV/B - 3	0.0015	56	0.16	-	N/A	SD
	5	49/m	MCL, Stage IV/A/S - 6	0.0015	28	0.073	-	n.d.	SD
	6	71/m	MCL, Stage IV/B/E - 6	0.0015	23	0.064	-	N/A	PD
	7	77/m	MCL, Stage IV/B/E/S - 1	0.0015	25	0.057	+[b]	N/A	SD
	8	65/m	CLL, Stage IV/B/ES - 9	0.0015	28	0.080	-	n.d.	PD
	9	75/m	FL, Stage II/B - 3	0.0015	28	0.13	-	N/A	SD
3	10	58/m	MCL, Stage III/B/S - 2	0.005	30	0.25	+	N/A	PD
	11	68/f	FL, Stage IV/B - 5	0.005	28	0.29	+	n.d.	SD
	12	65/m	MCL, Stage III/A/E - 1	0.005	30	0.27	+	N/A	SD
4[a]	13	60/m	SLL, Stage IV/B/S - 12	0.015	19	0.30	+	Complete	**PR** (1)
	14	73/m	MCL, Stage II/A/E - 2	0.015	28	0.77	+[b]	N/A	SD
	15	44/m	FL, Stage IV/B/ES - 3	0.015	56	1.62	+	Partial	**PR** (2)
	16	61/m	FL, Stage IV/A/S - 5	0.015	56	1.50	+	Complete	**CR** (7)
	17	67/m	MZL, Stage IV/B/S - 4	0.015	3	0.12	n.e.	N/A	n.e.
	18	64/m	FL, Stage IV/A/E - 4	0.015	31	0.87	+	N/A	PD
	19	75/m	MCL, Stage III/A - 3	0.015	2	0.025	n.e.	N/A	n.e.
	20	65/f	FL; Stage III/A/E - 6	0.015	52	1.37	+[b]	N/A	SD
	21	60/m	MCL, Stage IV/A/E - 3	0.015	53	1.46	+	None	SD
	22	67/f	FL, Stage IV/B - 1	0.015	58	1.32	+	Complete	MR
	23	67/m	DLBCL, Stage III/B/E - 7	0.015	5	0.17	n.e.	N/A	n.e.
	24	65/f	FL, Stage III/A - 6	0.015	39	0.99	+[b]	n.d.	SD
	25	74/f	WD, Stage IV/B - 5	0.015	56	1.49	+	Partial	SD
5	26	67/m	MCL, Stage IV/A - 7	0.03	53	3.5	+	Complete	SD
	27	48/m	FL, Stage III/A - 3	0.03	35	2.17	+	N/A	PD
	28	58/m	MCL, Stage III/A - 2	0.03	57	3.3	+	N/A	**CR** (13+)
	29	45/f	MCL, Stage IV/A/E - 6	0.03	30	1.4	+	Partial	PD
	30	59/m	MZL, Stage II/A/E - 2	0.03	2	0.12	n.e.	N/A	n.e.
	31	43/m	FL, Stage III/A - 2	0.03	56	3.53	+	N/A	MR
6[a]	32	72/m	MCL, Stage IV/A - 1	0.06	10	1.33	+	Complete	**PR** (8.5+)
	33	55/m	MCL, Stage IV/A/E - 3	0.06	30	3.7	+	Complete	**CR** (7+)
	34	52/m	FL, Stage IV/A - 3	0.06	57	7.77	+	N/A	**PR** (6.5+)
	35	47/m	FL, Stage III/A/S - 4	0.06	57	6.93	+	N/A	**PR** (2+)
	36	40/m	FL, Stage II/A - 1	0.06	32	3.44	+	N/A	**CR** (3+)
	37	70/m	FL, Stage III/A - 1	0.06	35	3.97	+	N/A	**PR** (2+)
	38	73/m	FL, Stage I/B - 1	0.06	57	6.36	+	N/A	**PR**[#] (<1+)

*Centrally confirmed complete (CR) and partial (PR) responses by Cheson criteria in bold; MR, minimal response (≥25 to <50%); SD, stable disease; PD, progressive disease; duration from first documentation of response in parentheses; + denotes an ongoing response until last assessment by June 1st, 2008; # response determined after the first 4 weeks of an eight-week treatment

[a]Cohort 2 was expanded to study a different methyl-prednisolone dose, cohort 4 to test three different schedules for first dosing, and cohort 6 for bridging to a new batch of clinical test material

[b]Very low initial B cell counts

n.e.: not evaluable because of treatment period <7 d

n.d.: not determined (infiltrated, but no second biopsy performed at end of treatment)

N/A: not assessable because no infiltration detected at study start

Table S2

Adverse Occuring in ³3 Patients Events Regardless of Causality (N=38)	Grade 1-4 N (%)
TOTAL	38 (100)
Lymphopenia	26 (68.4)
Pyrexia	26 (68.4)
Leukopenia	22 (57.9)
C-reactive protein increased	20 (52.6)
Headache	15 (39.5)
Thrombocytopenia	15 (39.5)
Chills	14 (36.8)
Fibrin D-dimer increased	14 (36.8)
Weight increased	14 (36.8)
Diarrhoea	13 (34.2)
Hepatic function abnormal	13 (34.2)
Neutropenia	13 (34.2)
Hypokalaemia	12 (31.6)
Anaemia	11 (28.9)
Fatigue	11 (28.9)
Haematuria	11 (28.9)
Alanine aminotransferase increased	10 (26.3)
Hyperglycaemia	10 (26.3)
Edema, peripheral	10 (26.3)
Weight decreased	10 (26.3)
Anorexia	9 (23.7)
Aspartate aminotransferase increased	9 (23.7)
Hypocalcaemia	8 (21.1)
Hypoproteinaemia	8 (21.1)
Activated partial thromboplastin time prolonged	7 (18.4)
Cough	7 (18.4)
Dizziness	7 (18.4)
Lipase increased	7 (18.4)
Proteinuria	7 (18.4)
Back pain	6 (15.8)
Blood glucose increased	6 (15.8)
Blood lactate dehydrogenase increased	6 (15.8)
Catheter site pain	6 (15.8)
Dyspnoea	6 (15.8)
Mucosal dryness	6 (15.8)
Nausea	6 (15.8)
Vomiting	6 (15.8)
Blood immunoglobulin A decreased	5 (13.2)
Blood immunoglobulin M decreased	5 (13.2)
Night sweats	5 (13.2)
Pleural effusion	5 (13.2)
Tremor	5 (13.2)
Abdominal pain	4 (10.5)
Asthenia	4 (10.5)
Confusional state	4 (10.5)
Constipation	4 (10.5)
Haemoglobin decreased	4 (10.5)
Hyperbilirubinaemia	4 (10.5)
Hypoalbuminaemia	4 (10.5)
Lymphocyte count decreased	4 (10.5)
Muscle spasms	4 (10.5)
Nasopharyngitis	4 (10.5)
White blood cell count decreased	4 (10.5)
Ascites	3 (7.9)
Blood immunoglobulin G decreased	3 (7.9)
Catheter site infection	3 (7.9)
Coagulation factor XIII level decreased	3 (7.9)
Hypercoagulation	3 (7.9)
Hyperhidrosis	3 (7.9)
Hypertension	3 (7.9)
Hyponatraemia	3 (7.9)
Insomnia	3 (7.9)
Liver disorder	3 (7.9)
Neutrophil count decreased	3 (7.9)
Edema	3 (7.9)
Pain in extremity	3 (7.9)
Pruritus	3 (7.9)

Table S3

Adverse Events of Grade 3 and 4 Regardless of Causality (N=38)	N (%)
TOTAL	36 (94.7)
Lymphopenia	26 (68.4)
C-reactive protein increased	13 (34.2)
Leukopenia	9 (23.7)
Neutropenia	6 (15.8)
Thrombocytopenia	6 (15.8)
Fibrin D dimer increased	5 (13.2)
Anaemia	4 (10.5)
Lymphocyte count decreased	4 (10.5)
Activated partial thromboplastin time prolonged	3 (7.9)
Hyponatraemia	3 (7.9)
White cell blood count decreased	3 (7.9)
Febrile neutropenia	2 (5.3)
Haemoglobin decreased	2 (5.3)
Hepatic function abnormal	2 (5.3)
Hyperglycaemia	2 (5.3)
Hyperuricaemia	2 (5.3)
Hypokalaemia	2 (5.3)
Neutrophil count decreased	2 (5.3)
Pyrexia	2 (5.3)
Abdominal pain	1 (2.6)
Anorexia	1 (2.6)
Aphasia	1 (2.6)
Apraxia	1 (2.6)
Asthenia	1 (2.6)
Blood alkaline phosphastase increased	1 (2.6)
Blood bilirubin increased	1 (2.6)
Blood calcium increased	1 (2.6)
Blood glucose increased	1 (2.6)
Blood immunoglobulin G decreased	1 (2.6)
Blood immunoglobulin M decreased	1 (2.6)
Blood potassium decreased	1 (2.6)
Cardiac failure	1 (2.6)
Cerebellar syndrome	1 (2.6)
Diabetes mellitus	1 (2.6)
Dyspnoea	1 (2.6)
Emotional distress	1 (2.6)
Encephalopathy	1 (2.6)
Fatigue	1 (2.6)
Gamma-glutamyltransferase increased	1 (2.6)
Gastroenteritis	1 (2.6)
Grand mal convulsion	1 (2.6)
Headache	1 (2.6)
Hepatic enzyme increased	1 (2.6)
Hyperbilirubinaemia	1 (2.6)
Hypertension	1 (2.6)
Hypocalcaemia	1 (2.6)
Hypophosphataemia	1 (2.6)
Hypotension	1 (2.6)
Liver disorder	1 (2.6)
Lobar pneumonia	1 (2.6)
Lymphoma	1 (2.6)
Metabolic acidosis	1 (2.6)
Mucosal erosion	1 (2.6)
Oedema peripheral	1 (2.6)
Pain	1 (2.6)
Pancreatitis	1 (2.6)
Pleural effusion	1 (2.6)
Soft tissue infection	1 (2.6)
Stasis dermatitis	1 (2.6)
Tremor	1 (2.6)

Legends for Supplementary Tables

Table S1. Demographics, treatment details and clinical outcome of patients treated in completed dose cohorts. Abbreviations used for diseases are: IC, immunocytoma; MCL, mantle cell lymphoma; CLL, chronic lymphocytic leukaemia; FL, follicular lymphoma, SLL, small lymphocytic leukaemia; MZL, mariginal zone lymphoma; DLBCL, diffuse follicular large B cell lymphoma; WD, Waldenstroem's disease. Stage of disease is defined according to Ann Arbor classification except for patient 1. Differences of total doses reflect differences in body surface area and treatment duration. B cell depletion is defined as absence of circulating B cells until end of treatment.

Table S2. Incidence of adverse events of grade 1-4 regardless of relationship to study drug thus far observed in ≥3 patients of completed dose cohorts during treatment with blinatumomab.

Table S3. Incidence of all adverse events of grade 3 and 4 regardless of relationship to study drug in patients of completed dose cohorts thus far observed during treatment with blinatumomab.