Immunopharmacological response of patients with B-lineage acute lymphoblastic leukemia to continuous infusion of T cell-engaging CD19/CD3-bispecific BiTE antibody blinatumomab

Running Title: Pharmacological response to blinatumomab infusion

Matthias Klinger,¹ Christian Brandl,¹ Gerhard Zugmaier,¹ Youssef Hijazi,¹ Ralf C. Bargou,² Max S. Topp,² Nicola Gökbuget,³ Svenja Neumann,⁴ Mariele Goebeler,² Andreas Viardot,⁵ Matthias Stelljes,⁶ Monika Brüggemann,⁴ Dieter Hoelzer,³ Evelyn Degenhard,¹ Dirk Nagorsen,¹ Patrick A. Baeuerle,¹* Andreas Wolf,¹ and Peter Kufer¹

¹Amgen Research (Munich) GmbH, Munich, Germany; ²Medizinische Klinik und Poliklinik II, Universitätsklinikum Würzburg, Würzburg, Germany; ³Medizinische Klinik II, Johann Wolfgang Goethe Universitätsklinikum, Frankfurt am Main, Germany; ⁴II. Medizinische Klinik und Poliklinik, Universitätsklinikum Schleswig-Holstein, Kiel, Germany; ⁵Klinik für Innere Medizin III, Universitätsklinikum Ulm, Ulm, Germany; and ⁶Medizinische Klinik A, Universitätsklinikum Münster, Münster, Germany

*Corresponding Author:
Patrick A. Baeuerle, Ph.D.
Amgen Research (Munich) GmbH, Staffelseestr. 2, 81477 Munich, Germany
E-mail: baeuerle@amgen.com
Tel.: +49-89-895277-601
Fax: +49-89-895277-84601
Scientific category: CLINICAL TRIALS AND OBSERVATIONS, LYMPHOID NEOPLASIA
Abstract

T cell-engaging CD19/CD3-bispecific BiTE antibody blinatumomab has shown an 80% complete molecular response rate and prolonged leukemia-free survival in patients with minimal residual B-lineage acute lymphoblastic leukemia (MRD+ B-ALL). Here, we report that lymphocytes in all patients of a phase 2 study responded to continuous infusion of blinatumomab in a strikingly similar fashion. After start of infusion, B cell counts dropped to less than one B cell/μl within an average of two days and remained essentially undetectable for the entire treatment period. By contrast, T cell counts in all patients declined to a nadir within less than one day and recovered to baseline within a few days. T cells then expanded and on average more than doubled over baseline within 2-3 weeks under continued infusion of blinatumomab. A significant percentage of reappearing CD8+ and CD4+ T cells newly expressed activation marker CD69. Shortly after start of infusion, a transient release of cytokines dominated by IL-10, IL-6 and IFN-γ was observed, which no longer occurred upon start of a second treatment cycle. The response of lymphocytes in leukemic patients to continuous infusion of blinatumomab helps to better understand the mode of action of this and other globally T cell-engaging antibodies. The trial is registered with ClinicalTrials.gov Identifier NCT00560794.
Introduction

Among all immune cells, cytotoxic T cells appear to have the highest potential for treatment of malignant diseases. This typically requires specific T cell clones recognizing tumor cell-associated peptide antigens in the context of MHC class I molecules. Multiple vaccination strategies, anti-CTLA4 antibodies boosting T cell responses, and adoptive transfer of ex vivo expanded, tumor-infiltrating lymphocytes have in many cases demonstrated the ability of tumor-specific T cells to treat late-stage cancers, albeit often with low response rates. Likewise, numerous mouse models have shown eradication of solid tumors by cytotoxic T cell responses and long-term protection from recurrence. Recently, two novel therapeutic modalities have gained clinical proof-of-concept for the treatment of lymphoma and leukemia in which cytotoxic T cells are globally engaged irrespective of their TCR specificity. One used the ectopic expression of a CD19-specific chimeric antigen receptor construct in transfected autologous T cells of patients. Patients with chronic lymphocytic leukemia (CLL) showed complete hematological responses after transfer of gene-modified, CD19-specific T cells. The other approach uses a CD19/CD3-bispecific T cell-engaging BiTE antibody called blinatumomab, which can transiently tether any cytotoxic T cell to CD19+ target B cells. Blinatumomab has shown high response rates in patients with non-Hodgkin lymphoma (NHL) and acute lymphoblastic leukemia (ALL). This BiTE antibody has preclinically been characterized in great detail and is currently tested in pivotal trials in ALL patients. Two other BiTE antibodies for treatment of solid tumors are currently in phase 1 clinical trials.

A recently published phase 2 study has shown a complete molecular response rate of 80% to monotherapy with blinatumomab in 20 evaluable MRD+ ALL patients. Recurrence or persistence of ALL in bone marrow – as detected by patient-specific PCR-based assays with a sensitivity down to 1 tumor cell in $10^4$ bone marrow cells – is among the most
important prognostic predictors for this disease. \(^{22-24}\) Conversion of patients into an MRD-negative status by blinatumomab predicted a positive outcome on long-term leukemia-free survival. \(^{16}\) In this trial, blinatumomab was continuously infused to patients by a portable mini-pump and port system at a dose level of 15 µg/m\(^2\)/day for 4 weeks. Subsequent treatment cycles were spaced by 2-week treatment-free intervals. The 15 µg-dose was found earlier to induce clearance of bone marrow infiltrations in NHL patients, whereas a dose of 60 µg/m\(^2\)/day was required for achieving a high response rate in lymphoma tissue in these diseases. \(^{15}\) Here, we analyzed all evaluable samples from the phase 2 study in order to understand the consequences of continuous and repeated exposure of MRD\(^+\) ALL patients to T cell-engaging BiTE antibody blinatumomab on (a) T cell kinetics, redistribution and behavior, (b) kinetics and durability of target B cell depletion, (c) kinetics and release of cytokines, and (d) serum concentrations of blinatumomab and PK parameters. We also assessed whether four non-responding patients had immunological parameters deviating from those of 16 responding patients.

We here report that T and B lymphocytes in a defined population of MRD\(^+\) ALL patients showed a highly predictable behavior upon start and during continuous intravenous (i.v.) infusion of blinatumomab. The observed immunopharmacological reactions are consistent with the mode of action of a T cell-engaging antibody with the potential to globally activate T cells in a target cell-dependent fashion. The data also explain why the continuous infusion of blinatumomab is well tolerated and in that allows for a tight control over T cell activation. Lastly, the lymphocyte response to blinatumomab of 16 responding patients did not significantly differ from that of four non-responding patients.
Patients and methods

Patient characteristics

Detailed patient characteristics have been published elsewhere.\textsuperscript{16} Adult patients with B-lineage ALL in hematologic complete remission (CR) were eligible if they expressed the precursor B phenotype and were either molecularly refractory or had a molecular relapse with quantifiable MRD load of $\geq 1 \times 10^{-4}$ starting at any time point after consolidation I of front-line therapy within GMALL protocols. Of 21 patients enrolled into this study, 14 had individual TCR/Ig gene rearrangements, 5 presented with a BCR-ABL and 2 with an MLL-AF4 translocation. Twenty patients completed at least one treatment cycle and were assessable for pharmacokinetic and pharmacodynamic parameters. All patients enrolled into this study gave written informed consent. The clinical study was approved by the local ethics committees of the participating university hospitals and the responsible authority of the Federal Republic of Germany and was conducted in accordance with the provisions of the Declaration of Helsinki and Good Clinical Practice guidelines. The trial is registered with ClinicalTrials.gov Identifier NCT00560794.

Study design and drug administration

The study was designed as an open-label, multicenter, single-arm, phase 2 clinical trial that investigated the efficacy, safety and tolerability, and pharmacokinetics and pharmacodynamics of blinatumomab.\textsuperscript{16} Using a portable mini-pump and port system, patients received blinatumomab as continuous intravenous (i.v.) infusion at a dose of 15 µg/m²/day over a 4-week cycle followed by a treatment-free period of 2 weeks. Patients were treated as out-patients and only hospitalized for the first two to three days of each treatment cycle. Responders were permitted to receive up to three additional consolidation cycles of treatment with blinatumomab.\textsuperscript{16}
**Measurement of response**

The primary end point of this study was defined by the incidence of MRD negativity (i.e., $<1 \times 10^{-4}$) within seven treatment cycles with blinatumomab. Quantitative PCR for individual TCR/Ig gene rearrangements, BCR-ABL and MLL-AF4 translocations was performed as described.\(^{16}\) MRD levels were quantified and reassessed after each treatment cycle. Detection of MRD negativity was confirmed by a second bone marrow biopsy taken within 2 weeks after the first one.

**PK assay and analysis**

Blood samples for the determination of serum concentrations of blinatumomab were taken at different time points during infusion and after infusion stop to allow determination of PK parameters. Quantification of blinatumomab in human serum samples was performed as described previously.\(^{15}\) Briefly, the assay is based on CD69 up-regulation on the surface of newly activated T cells upon dual binding of blinatumomab to T cells and Raji lymphoma cells. Expression of CD69 is monitored by FACS analysis and is increased in a drug dose-dependent manner. The lower limit of quantification (LLOQ) of the assay was 100 pg/ml. The average steady state serum concentration ($C_{ss}$) in an individual patient was calculated from available and reliable data points at plateau in each cycle. The systemic clearance ($CL$) was calculated according to the equation $CL = Ro/C_{ss}$, where $Ro$ is the dosing rate in $\mu g/m^2/day$. Other PK parameters such as elimination half-life ($T_{1/2,\beta}$), volume of distribution ($V_Z$), and area under the serum concentration-time curve to 28 days and to infinity (AUC $0-28$ and AUC $0-inf$, respectively) were estimated by applying standard non-compartmental methods using WinNonlin software (Pharsight, Sunnyvale, CA, USA).
Cytokine assay

Serum concentrations of six different cytokines (IL-2, IL-4, IL-6, IL-10, IFN-γ and TNF-α) were assessed using a commercially available FACS-based cytometric bead array (CBA) kit (Becton Dickinson, Heidelberg, Germany). The assay was used according to the manufacturer’s protocol and was internally validated for the intended use. The limit of detection (LOD) for the cytokine determination was 20 pg/ml, the LLOQ was 125 pg/ml.

Analysis of lymphocyte subpopulations

Lymphocytes in patients’ peripheral blood were analyzed by FACS and quantified as described previously. Briefly, blood samples were drawn at 0.75, 2, 6, 12, 24, 30 and 48 hours after the start of treatment as well as on treatment days 7, 14, 21 and 28 plus 1 week after the end of treatment. Peripheral blood mononuclear cells (PBMC) were isolated by an adapted Ficoll density gradient separation and subsequently stained with fluorescence-labelled antibodies against cell surface or intracellular markers as follows: B cells: CD19; B cell apoptosis: annexin V; T cells: γδ TCR, CD3, CD4, CD8, CD56; T cell activation: CD69, CD25, HLA-DR; CD8+ T cell subsets: CD45RA, CD28, CD197; and CD4+ regulatory T cells: CD25, Foxp3. FACS data collection was conducted on a FACSCalibur or FACSCanto II instrument (Becton Dickinson) and statistical analysis was performed either with the software CellQuest Pro (Becton Dickinson) or FCS Express (De Novo Software, Los Angeles, CA, USA). Finally, percentage values of lymphocyte subpopulations were correlated with the absolute lymphocyte number of a differential blood count to calculate the respective absolute subpopulation numbers. The overall AUC was determined as the area under the αβ T cell curve of the entire 4-week treatment period; and the baseline AUC as the corresponding area above the respective baseline αβ T cell count.
Statistical analysis

Depending on whether means or medians were compared, a paired t-test or a Wilcoxon matched-pairs signed rank test/Mann-Whitney test was used for statistical analysis in Prism (GraphPad Software, La Jolla, CA, USA). A two-tailed p-value <0.05 was considered statistically significant.
Results

Steady state serum concentrations of continuously infused blinatumomab are very stable during repeated 4-week treatment periods

Blinatumomab was continuously i.v. administered for 4-week infusion periods to MRD⁺ ALL patients via an implanted port system using a portable mini-pump. One out of 21 patients enrolled into this study was not evaluable for response due to early dropout. Patient characteristics have been reported elsewhere. In order to determine levels of biologically active BiTE antibody in serum samples of patients, a sensitive PK bioassay was developed with a lower limit of quantification of 100 pg/ml in human serum. PK parameters from the first 4-week treatment cycle are shown in Table 1. The steady state serum concentration (Css) across all assessable patients ranged from 492-1,050 pg/ml with a mean (±SD) of 731 ±163 pg/ml. The elimination serum half-life of blinatumomab (T_{1/2, β}) was on average (±SD) 1.25 ±0.63 hours. The clearance (CL) was on average (±SD) 22.3 ±5.0 l/day/m², and the volume of distribution (V₁) 1.61 ±0.74 l/m². The average Cₛₛ of evaluable patients over four 4-week treatment cycles, each followed by a 2-week treatment-free interval, are shown in Figure 1. Mean Cₛₛ for cycles 2, 3 and 4 were statistically not different from the average of cycle 1. The two-tailed p-value for cycle 1 relative to cycle 4 was 0.4334. No human anti-mouse antibody (HAMA) response to blinatumomab was detected in any of the patients.

Blinatumomab causes sustained depletion of peripheral B cells

We next studied in blood samples from MRD⁺ ALL patients the effect of blinatumomab on counts of peripheral B cells, which are targets for BiTE-engaged T cells. In vitro co-culture experiments have shown that blinatumomab can induce redirected lysis of CD19⁺ B lymphocytes and malignant B cell lines by previously resting peripheral T cells at sub-pM
Patients presented with a wide range of B cell counts at baseline ranging from 2 to 454 B cells/µl (mean ±SD was 75 ±119 B cells/µl) (Tab. 2). Half of the patients had low B cell counts ≤20 cells/µl at baseline, which most likely resulted from previous treatment with standard chemotherapy (Fig. 2A). Upon start of continuous infusion of blinatumomab, the majority of patients showed a decline of B cells to the limit of detection of 1 cell/µl within less than 1 day. B cell counts dropped below the detection limit within a mean (±SD) of 2.18 ±3.80 days (range from 0.03 to 13.94 days) (Fig. 2B). B cell counts remained below the detection limit for the entire treatment period as shown for cycle 1 (Fig. 2B and suppl. Fig. S1A) and cycle 2 (suppl. Fig. S1D). Apoptotic B cell death and not B cell redistribution was the most likely reason for a sustained absence of B cells during continuous blinatumomab infusion. This is suggested by increased binding of annexin V, an early apoptosis marker, to B cells as exemplified for one patient (Fig. 2C). Annexin V staining of B cells was only feasible in those patients with high initial and slowly declining B cell counts. Of note, peripheral B cell counts did not detectably recover during or in between up to seven analyzed treatment cycles (suppl. Fig. S1A,D; data not shown for cycles 3 to 7).

Blinatumomab induces a swift decline of peripheral T cell counts upon each infusion start followed by recovery

At baseline, MRD+ ALL patients presented with a mean (±SD) of 532 ±283 T cells/µl (range from 112 to 1,364 T cells/µl) (Tab. 2). A peculiar reaction of peripheral T cells in response to the start of blinatumomab infusion was a fast drop of their counts within the first hours of exposure to the BiTE antibody (Fig. 3A-B). A nadir with a mean count (±SD) of 36 ±49 T cells/µl (range from 2 to 197 T cells/µl) was reached within 0.36 ±0.24 days (range from 0.06 to 1.09 days). In support of a redistribution phenomenon, T cell counts recovered and reached 50% of baseline counts after a mean (±SD) of 3.13 ±1.85 days (range from 0.82 to 9.10 days), and were back to baseline levels after 8 to 9 days (Fig. 3A). The initial eclipse of
T cells was observed for every patient and was of high statistical significance ($p = 0.0001$) (Fig. 3B). In the second treatment cycle, every patient again showed a temporary eclipse of T cells upon start of blinatumomab infusion with an accelerated recovery to baseline compared to cycle 1 (suppl. Fig. S1A,D). This redistribution phenomenon was also observed in all subsequent treatment cycles with similar kinetics to those in cycle 2 (data not shown).

**Blinatumomab induces an expansion of the peripheral T cell compartment during the first treatment cycle**

Following the initial eclipse, T cell counts not only returned to baseline in all patients under continued treatment with blinatumomab but in all but one patient exceeded baseline levels (Fig. 3A,C). A maximal expansion of T cells during the 4-week infusion period was reached after a mean ($\pm$SD) of 17.57 ±9.00 days (range from 2.06 to 28.06 days) (Tab. 2) with T cells increasing with high significance ($p = 0.0002$) relative to baseline (= 100%) to a mean ($\pm$SD) of 220 ±123% (range from 93 to 547%).

Individual $\alpha\beta$ T cell responses for all evaluable patients are depicted in supplementary Figure S1A. Analysis of T cell subpopulations showed that in 9 out of 20 patients CD4$^+$ T cells expanded stronger than CD8$^+$ T cells, in 4 out of 20 patients CD8$^+$ T cells more than CD4$^+$ T cells, and in 7 out of 20 patients more or less equally. Only in one patient (a responder), the CD4$^+$ T cell expansion also involved an increase of CD4$^+$ regulatory T cells while otherwise CD4$^+$ regulatory T cells stayed at very low counts throughout blinatumomab treatment (suppl. Fig. S1B). A major contribution to the number of expanding T cells was made by effector memory T (T$_{EM}$) cells of the CD45RA/CD197$^-$ phenotype as shown for CD8$^+$ T cells (suppl. Fig. S1C). T$_{EM}$ cells expressing CD45RA (T$_{EMRA}$ cells) also detectably contributed to expanding T cells.

Eleven out of 14 patients who were available for pharmacodynamic analysis of their second treatment cycle after a 2-week break still had T cell counts higher than at baseline of
the first cycle. However, in 11 out of these 14 patients T cells did not further expand above baseline of the second cycle after recovery from the initial eclipse (suppl. Fig. S1D). No further T cell expansion was observed in all subsequent treatment cycles (data not shown).

**Blinatumomab increases the percentage of activated peripheral CD8+ and CD4+ T cells**

In order to explore causes for a transient redistribution of a large proportion of peripheral T cells in response to blinatumomab (Fig. 3), we investigated the activation state of reappearing T cells by FACS-based analysis of the surface expression of immediate early activation marker CD69, and of late T cell activation markers CD25 and HLA-DR. Most consistent data were obtained with CD69, which are here shown for both CD8+ and CD4+ T cells (Fig. 4A and B, respectively). In all assessable patients, the percentage of CD69+ peripheral T cells did increase with peak activation occurring within the first 48 hours after start of infusion. The percentage of CD8+/CD69+ T cells maximally increased from a median of 19.47% (range from 9.66 to 34.43%) to 48.78% (range from 17.77 to 78.61%), and for CD4+/CD69+ T cells from a median of 12.32% (range from 0.40 to 49.71%) to 35.63% (range from 8.42 to 79.15%). Both increases were statistically highly significant (p = 0.0001). In conclusion, blinatumomab induced an increase of the percentage of activated circulating T cells consistent with a polyclonal T cell activation by the CD3-engaging BiTE antibody.

**T cell parameters of responding patients are not different from those of non-responding patients**

In this phase 2 study, 4 out of 20 evaluable MRD+ ALL patients did not respond by a complete molecular response to treatment with blinatumomab monotherapy. Here, we assessed whether T cell parameters of non-responding patients were different from those of responding patients. Retrospective statistical analysis of baseline T cell counts (Fig. 5A), maximally reached T cell counts (Fig. 5B), or T cell exposure by baseline (Fig. 5C) or overall
area under the αβ T cell curve (Fig. 5D) did not significantly correlate with response (p = 0.6149, p = 0.9623, p = 0.6956 and p = 0.8668, respectively). Obviously, non-responding patients had similar levels, exposure to and reactivity of T cells as responders. The load of tumor cells in bone marrow and counts of peripheral target B cells did likewise not correlate with response (Fig. 5E-F; p = 0.1079 and p = 0.9554, respectively). From these analyses, it did not appear that T or B cell parameters, or tumor load in bone marrow can provide an explanation for the non-responsiveness of 4 patients to blinatumomab in this phase 2 study.

*Start of blinatumomab infusion causes a transient release of cytokines which does not recur upon start of a second treatment cycle*

Concentrations of selected cytokines known to be released by activated T cells were measured in serum samples from MRD⁺ ALL patients all of whom received glucocorticoid coverage upon start of each treatment cycle. IL-2, IL-6, IL-10, IFN-γ and TNF-α were detectable during the first two days after start of infusion with maximal levels reached during the first day (Fig. 6A). IL-4 was not detectable. Highest cytokine levels were observed for IL-10, followed by IL-6 and IFN-γ with average serum peak concentrations (±SD) of 864 ±1,201, 479 ±735, and 422 ±599 pg/ml, respectively. TNF-α and IL-2 reached lower peak concentrations which were on average (±SD) at 190 ±213 and 99 ±79 pg/ml, respectively. After two days, cytokine levels declined quickly and were barely detectable in sera of patients later during treatment. Upon start of a second treatment cycle, a robust cytokine release was no longer detectable (Fig. 6B). Of note, cytokine responses showed a high inter-patient variability, and approximately half of the patients showed hardly any release of cytokines in cycle 1 (Fig. 7). The magnitude of the cytokine response did not significantly correlate with target B cell frequency in blood or bone marrow (data not shown).

The peak levels of transiently released cytokines IL-2, IL-6, IL-10, IFN-γ or TNF-α did not correlate with a clinical response to blinatumomab monotherapy (Fig. 7A-E; p =
0.2343, p = 0.3202, p = 0.8133, p = 0.2356, and p = 0.3107, respectively). Likewise, a transient increase in body temperature observed at treatment start showed no correlation with response (Fig. 7F; p = 0.2761).
Discussion

This is the first study investigating in detail in leukemic patients the pharmacological and pharmacodynamic effects of continuous i.v. infusion of the globally T cell-activating CD3/CD19-bispecific BiTE antibody blinatumomab. The continuous i.v. dosing regimen has produced a high response rate of 80% in a clinical phase 2 study in MRD+ ALL patients. At the time of revision of this manuscript, four out of 10 non-transplanted MRD-negative responders are still in remission corresponding to a progression free survival of up to 38 months. Here, we analyzed the set of 20 response-evaluable patients from this study for pharmacokinetics, response of B and T cells, and cytokine release during blinatumomab administration.

Key observations were the following: (1) Continuous i.v. infusion of blinatumomab gave rise to a mean steady state serum concentration in patients of 0.73 ng/ml (13 pM) that did not change in up to four treatment cycles. (2) As seen in in vitro co-culture experiments, blinatumomab caused a durable depletion of CD19-expressing target B cells in patients by redirected lysis. (3) Upon start of infusion, peripheral T cell counts swiftly dropped but recovered within a few days, and then expanded above baseline counts under treatment. (4) A large proportion of recovering T cells up-regulated the activation marker CD69. (5) Cytokines were transiently released only upon start of the first infusion with a high inter-patient variability, and were no longer detected upon start of a second treatment cycle. Of note, most of the pharmacokinetic and pharmacodynamic effects of blinatumomab showed very little variation in the MRD+ ALL patient population, which was characterized by a low load of target B cells in blood and bone marrow. Months of constant exposure of patients to the globally T cell-activating BiTE antibody blinatumomab did neither lead to signs of uncontrolled T cell activation (e.g., a cytokine storm) nor to signs of T cell anergy, as could have been evident from the recovery of target B cell counts under continued treatment.
Continuous i.v. infusion of blinatumomab led to stable and comparable steady state levels in MRD\(^+\) ALL patients within each 4-week treatment period. This indicates that blinatumomab was not significantly sequestered, for instance, by binding to CD19 on target B cells or to CD3 on T cells. At mean serum concentrations of only 0.013 nM and equilibrium binding constants of blinatumomab for the CD19 and CD3 antigens of ca. 1 and 100 nM, respectively,\(^{17}\) significant cell binding of the BiTE antibody is also not expected from a theoretical position. Comparable steady state levels also indicate that blinatumomab was not sequestered by a neutralizing antibody response to the BiTE antibody, which is consistent with a low immunogenicity rate of <1% observed across all trials with blinatumomab.\(^{15,16}\)

Steady state levels were rapidly achieved after start of infusion and – due to the short serum half-life of the drug – serum concentrations of blinatumomab declined quickly after stop of infusion. In case of severe adverse events, the latter can provide for an effective means to control drug exposure by health care practitioners. The short serum half-life and relatively high clearance of blinatumomab are consistent with a renal elimination of the 55 kDa antibody construct lacking an Fc domain, which mediates the long serum half-life of regular IgG antibodies.\(^{27}\)

The decline of peripheral B cells upon start of blinatumomab infusion below the limit of detection was most likely the consequence of their redirected lysis. This is not only consistent with observations in in vitro co-cultures,\(^{17,25}\) where comprehensive redirected lysis of CD19\(^+\) target cells by BiTE-engaged T cells is detected at the same blinatumomab concentrations reached in patients, but also with binding of annexin V to declining B cells of MRD\(^+\) ALL patients, an effect that had also been noticed earlier in NHL patients.\(^{15}\) Induction of apoptosis after BiTE-mediated synapse formation by activation of caspases 3/7 in target cells was also demonstrated for other BiTE antibodies to be relevant for efficient cytotoxicity.\(^{28}\) The observation that B cell counts did not detectably recover under continued BiTE treatment for up to seven 4-week cycles suggests that T cells in the presence of
blinatumomab were continuously active in depleting new B cells derived from CD19− pluripotent stem cells.

The majority of adverse events of blinatumomab in MRD+ ALL patients was recorded during the first days following start of blinatumomab infusion.16 Most frequent were flu-like symptoms such as pyrexia, headache, chills and fatigue, which predominantly were below CTC grade 3. As reported here, these side effects coincided with the maximal increase of activated CD8+ and CD4+ T cells within two days after start of infusion, and the maximal release of cytokines during the first day of infusion. Likewise, the eclipse of T cells upon treatment start fell into this early phase of T cell activity. The observation that T cell activation, redistribution and cytokine release were all transient under continued treatment with blinatumomab suggests an adaptive reaction of newly activated T cells, which is also known as “first dose effect”. In vitro co-culture experiments have also shown that cytokine release and CD69 up-regulation by BiTE-activated T cells are transient.17,20,25 Of note, both are not necessary for redirected lysis of target B cells as it continues for days after cytokine release and CD69 expression have ceased. The expansion of peripheral T cells above baseline counts may be explained by either proliferation or redistribution of T cells from tissues to blood, or both. The increase of T cell counts during treatment week two and three was dominated by an expansion of the TEM and TEMRA subsets in most patients. This selective response of memory T cells to BiTE stimulus may be due to less stringent requirements or lower thresholds for full activation because of differences in employed signaling pathways compared to naïve T cells.29,30 The expansion of T cells in itself may be considered beneficial for these heavily immunocompromised patients.

While T cell redistribution was observed with the start of each treatment cycle, a release of cytokines and an expansion of peripheral T cells were confined to the first cycle. This indicates that the extent of cytokine release and T cell expansion may correlate with target B cell load while the initial T cell redistribution is independent thereof. This
redistribution is likely due to an increased adhesion of T cells to the blood endothelium which appears to be triggered by monovalent binding of blinatumomab to CD3. However, monovalent binding of blinatumomab does not activate T cells which is strictly dependent on the presence of target cells.\textsuperscript{31} In vitro co-culture experiments with endothelial cells are ongoing to investigate in more detail the redistribution phenomenon of T cells upon start of BiTE treatment.

Four out of 20 evaluable MRD\textsuperscript{+} ALL patients did not respond to blinatumomab treatment with a complete molecular response.\textsuperscript{16} These four patients had a rather low tumor cell load in their bone marrow, showed with the exception of IL-10 rather low cytokine responses, but had average T cell counts and an average responsiveness of T cells to the BiTE antibody. Moreover, many responders had no detectable pro-inflammatory cytokine release, high IL-10 levels as well, and much lower T cell counts and exposure than the four non-responders. Based on the present data set we could not identify an immunological or pharmacological parameter that would correlate with the response of MRD\textsuperscript{+} ALL patients to blinatumomab treatment. This non-responsiveness could be caused by yet to be defined differences in the aggressiveness of the underlying leukemia. Hampered T cell activity by tyrosine kinase inhibitor co-medication or reduced accessibility to target cells might also be of importance. Lastly, inherent resistance mechanisms of target cells may involve protection from perforin/granzyme B, alterations of the caspase pathway or outgrowth of CD19\textsuperscript{−} leukemic cell clones. Future studies will have to investigate in more detail the cellular mechanisms underlying a potential induction of resistance to blinatumomab treatment.
Acknowledgments

The authors would like to thank Elke Burghart, Sandra Wissing and Christiane Simmich for excellent technical support and Metronomia Clinical Research GmbH for data compilation.

Authorship

Contribution: M.K. designed and performed research, analyzed and interpreted data, and edited the manuscript; C.B. performed research; Y.H. performed pharmacokinetic analysis; N.G., S.N., M.G., A.V. and M.S. procured patient samples and collected clinical data; M.B. analyzed MRD status; G.Z., R.C.B., M.S.T., N.G., D.H., E.D. and D.N. were involved in trial design and study conduct; P.A.B. wrote the manuscript; and A.W. and P.K. designed research and analyzed and interpreted data.

Conflict-of-interest disclosure: M.K., C.B., G.Z., Y.H., E.D., D.N., P.A.B., A.W. and P.K. are employees of Amgen, and have ownership interests; R.C.B., M.S.T., N.G., S.N., M.G., A.V., M.S. and M.B. received research funding from Amgen; R.C.B., M.S.T. and D.H. have consultancy agreements with Amgen and are members of its advisory committees.

Correspondence: Patrick A. Baeuerle, Amgen Research (Munich) GmbH, Staffelseestr. 2, 81477 Munich, Germany; e-mail: baeuerle@amgen.com.
References


# Tables

Table 1. Pharmacokinetic parameters of continuously i.v. infused blinatumomab in the first treatment cycle of MRD+ ALL patients

<table>
<thead>
<tr>
<th></th>
<th>( \text{AUC}_{0-28} ), day*pg/ml</th>
<th>( \text{AUC}_{0-inf} ), day*pg/ml</th>
<th>( \text{Css} ), pg/ml</th>
<th>( T_{1/2, \beta} ), h</th>
<th>( CL ), l/day/m²</th>
<th>( V_Z ), l/m²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>19,659</td>
<td>19,764</td>
<td>731</td>
<td>1.250</td>
<td>22.3</td>
<td>1.61</td>
</tr>
<tr>
<td>±SD</td>
<td>±4,413</td>
<td>±4,474</td>
<td>±163</td>
<td>±0.634</td>
<td>±5.0</td>
<td>±0.74</td>
</tr>
<tr>
<td>Range</td>
<td>12,864 – 27,979</td>
<td>12,914 – 28,135</td>
<td>492 – 1,050</td>
<td>0.52 – 2.76</td>
<td>15.2 – 32.5</td>
<td>0.87 – 3.27</td>
</tr>
</tbody>
</table>

AUC indicates area under the curve; \( \text{Css} \), steady state serum concentration; \( T_{1/2, \beta} \), terminal serum half-life; \( CL \), systemic clearance; and \( V_Z \), distribution volume.
### Table 2. Pharmacodynamic effects of continuously i.v. infused blinatumomab on T and B cell kinetics in the first treatment cycle of MRD+ ALL patients

<table>
<thead>
<tr>
<th></th>
<th>Mean ±SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>T Cells</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Time, days</td>
<td></td>
</tr>
<tr>
<td>Time to T cell nadir</td>
<td>0.36 ±0.24</td>
<td>0.06 – 1.09</td>
</tr>
<tr>
<td>Time to 50% T cell recovery</td>
<td>3.13 ±1.85</td>
<td>0.82 – 9.10</td>
</tr>
<tr>
<td>Time to maximal T cell increase</td>
<td>17.57 ±9.00</td>
<td>2.06 – 28.06</td>
</tr>
<tr>
<td></td>
<td>T cells, per μl</td>
<td></td>
</tr>
<tr>
<td>T cell count at baseline</td>
<td>532 ±283</td>
<td>112 – 1,364</td>
</tr>
<tr>
<td>T cell count at nadir</td>
<td>36 ±49</td>
<td>2 – 197</td>
</tr>
<tr>
<td>Maximal T cell count during treatment</td>
<td>1,013 ±430</td>
<td>376 – 1,954</td>
</tr>
<tr>
<td>T cell count at end of treatment</td>
<td>737 ±317</td>
<td>152 – 1,410</td>
</tr>
<tr>
<td>T cell count after 1-week recovery period</td>
<td>825 ±432</td>
<td>287 – 1,599</td>
</tr>
<tr>
<td></td>
<td>T cells, %</td>
<td></td>
</tr>
<tr>
<td>Maximal T cell increase relative to baseline</td>
<td>220 ±123</td>
<td>93 – 547</td>
</tr>
<tr>
<td><strong>B Cells</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Time, days</td>
<td></td>
</tr>
<tr>
<td>Time to B cell depletion</td>
<td>2.18 ±3.80</td>
<td>0.03 – 13.94</td>
</tr>
<tr>
<td></td>
<td>B cells, per μl</td>
<td></td>
</tr>
<tr>
<td>B cell count at baseline</td>
<td>75 ±119</td>
<td>2 – 454</td>
</tr>
<tr>
<td>B cell count at end of treatment</td>
<td>1 ±1</td>
<td>0 – 4</td>
</tr>
<tr>
<td>B cell count after 1-week recovery period</td>
<td>1 ±3</td>
<td>0 – 13</td>
</tr>
</tbody>
</table>

Mean values ±SD and ranges for 20 evaluable patients are shown.
Figure legends

Figure 1. Mean steady state serum concentrations of continuously i.v. infused blinatumomab in up to four consecutive treatment cycles. C_{ss} were calculated for the indicated number of evaluable patients per cycle and the mean C_{ss} ± SD is shown for each cycle. Denominators give the total number of treated patients in each cycle. The p-value for the non-significant difference between cycle 1 and 4 is depicted by a bracket. Likewise, all other cycles did not differ significantly among each other. C_{ss} indicates steady state serum concentration.

Figure 2. Effect of continuously i.v. infused blinatumomab on peripheral B cell counts and apoptosis during the first treatment cycle of MRD⁺ ALL patients. (A) Individual B cell counts of 20 evaluable patients are shown at baseline and at first occurrence of B cell depletion ≤ 1 cell/µl. (B) Mean B cell counts are shown at baseline, at the mean nadir of ≤ 1 cell/µl, at the end of the 4-week treatment period, and at 1 week after the end of infusion. Error bars indicate SD for the respective cell counts and time points. (C) Binding of annexin V to B cells of one patient with slowly declining peripheral B cell counts. MFI indicates median fluorescence intensity as determined by FACS staining.

Figure 3. Effect of continuously i.v. infused blinatumomab on peripheral T cell counts during the first treatment cycle of MRD⁺ ALL patients. (A) Mean T cell counts of 20 evaluable patients are shown at baseline, at the mean nadir of the initial redistribution, at the mean time point of 50% recovery to baseline, at the mean time point of maximal expansion, at the end of the 4-week treatment period, and at 1 week after the end of infusion. Error bars indicate SD for the respective cell counts and time points. Numbers of this graph are given in Table 2. (B) Statistical analysis of the initial T cell redistribution observed during the first day
and (C) of the maximal T cell expansion during blinatumomab infusion. Bars in the respective left subpanels give median values, brackets indicate p-values. Individual patient correlations are shown in the respective right subpanels.

Figure 4. Effect of continuously i.v. infused blinatumomab on the activation of peripheral CD8⁺ and CD4⁺ T cells during the first treatment cycle of MRD⁺ ALL patients. The expression of the immediate early activation marker CD69 on the surface of gated (A) CD8⁺ and (B) CD4⁺ T cell subpopulations was determined by FACS at baseline and throughout treatment, and the percentages of activated, CD69⁺ T cell subpopulations were calculated. Values of (A) 14 and (B) 15 assessable patients are shown at baseline and at the time point of maximal T cell activation which occurred within the first two days after infusion start for all patients. Bars in the respective left subpanels give median percentages, brackets indicate p-values. Individual patient correlations are shown in the respective right subpanels.

Figure 5. Correlation of T and B cell parameters and MRD level with clinical response to blinatumomab treatment in MRD⁺ ALL patients. 16 patients responding with a complete molecular response in bone marrow after the first treatment cycle are compared to four non-responders. Statistical analyses are shown for (A) T cell counts at baseline, (B) maximal T cell counts reached during expansion, (C) the area under the T cell curve above the respective baseline T cell count, (D) the overall area under the T cell curve regardless of the respective baseline T cell count, (E) target B cell counts at baseline, and (F) the tumor load in the bone marrow before treatment as determined by patient-specific PCR. Bars give median values, brackets indicate p-values. AUC indicates area under the curve and is used as a measure of T cell exposure.
Figure 6. Effect of continuously i.v. infused blinatumomab on cytokine release into sera of MRD+ ALL patients. Serum samples of assessable patients were analyzed for concentrations of IL-2, IL-4, IL-6, IL-10, IFN-γ and TNF-α at the indicated time points. Mean values are shown for the (A) first and (B) second treatment cycle. IL-4 concentrations were below the limit of detection throughout all patients and are not depicted.

Figure 7. Correlation of peak cytokine concentrations and body temperature with clinical response to blinatumomab treatment in MRD+ ALL patients. 16 patients responding with a complete molecular response in bone marrow after the first treatment cycle are compared to four non-responders. All depicted values represent individual (A-E) peak cytokine concentrations or (F) peak body temperatures within the first three days after infusion start. Statistical analyses are shown for (A) IL-2, (B) IL-6, (C) IL-10, (D) IFN-γ, (E) TNF-α, and (F) body temperature. IL-4 concentrations were below the limit of detection throughout all patients and were not analyzed. Bars give median values, brackets indicate p-values.
Figure 2

A  B Cell Depletion (to ≤1/μl)

B  B Cell Kinetics

C  B Cell Apoptosis

N = 20
Figure 3

T Cell Kinetics

αβ T Cells [10^3/μL]

0.0 0.3 0.6 0.9 1.2 1.5

0 7 14 21 28 35

Time [Days]

N = 20
Figure 3

B

T Cell Redistribution

\[ p = 0.0001 \]

\( \alpha\beta \) T Cells [10^3/μL]

C

T Cell Expansion

\[ p = 0.0002 \]
A

CD8⁺ T Cell Activation

B

CD4⁺ T Cell Activation
Figure 5

A. Baseline T Cell Counts

B. Maximal T Cell Counts

C. Baseline AUC

D. Overall AUC

E. Baseline B Cell Counts

F. MRD Level
Figure 7

A. IL-2

B. IL-6

C. IL-10

D. IFN-γ

E. TNF-α

F. Body Temperature

For personal use only.
Immunopharmacological response of patients with B-lineage acute lymphoblastic leukemia to continuous infusion of T cell-engaging CD19/CD3-bispecific BiTE antibody blinatumomab

Matthias Klinger, Christian Brandl, Gerhard Zugmaier, Youssef Hijazi, Ralf C. Bargou, Max S. Topp, Nicola Gökbuget, Svenja Neumann, Mariele Goebeler, Andreas Viardot, Matthias Stelljes, Monika Brüggemann, Dieter Hoelzer, Evelyn Degenhard, Dirk Nagorsen, Patrick A. Baeuerle, Andreas Wolf and Peter Kufer