A novel Fc-engineered monoclonal antibody to CD37 with enhanced ADCC and high pro-apoptotic activity for treatment of B-cell malignancies

Short Title: Therapeutic potential of an Fc-engineered CD37 antibody

Karl-Heinz Heider,1 Kerstin Kiefer,1 Thorsten Zenz,2 Matthias Volden,2 Stephan Stilgenbauer,2 Elinborg Ostermann,1 Anke Baum,1 Herbert Lamche,1 Zaruhi Küpcü,1 Alexander Jacobi,3 Steffen Müller,3 Ulrich Hirt,1 Günther R. Adolf,1 Eric Borges1

1Boehringer Ingelheim RCV, Vienna, Austria; 2University of Ulm, Ulm, Germany; 3Boehringer Ingelheim Pharma, Biberach/Riss, Germany.

*present address: Department of Translational Oncology, National Center for Tumor Diseases (NCT) and German Cancer Research Center, Im Neuenheimer Feld 460, Heidelberg, Germany. Department of Medicine V, University of Heidelberg, Heidelberg, Germany

Corresponding author:

Karl-Heinz Heider
Boehringer Ingelheim RCV GmbH & Co KG
Dr. Boehringer Gasse 5-11
Vienna
Austria
Tel: +43 1-80105-2329
Fax: +43 1-80105-32329
E-mail: karl-heinz.heider@boehringer-ingelheim.com
Abstract

The tetraspanin CD37 is widely expressed in B-cell malignancies and represents an attractive target for immunotherapy with monoclonal antibodies. We have chimerized a high-affinity mouse antibody to CD37 and engineered the CH2 domain for improved binding to human Fcγ receptors. The resulting mAb 37.1 showed high intrinsic pro-apoptotic activity on malignant B-cells accompanied by homotypic aggregation. Furthermore, the antibody mediated high antibody-dependent cell-mediated cytotoxicity (ADCC) on lymphoma and primary CLL cells. mAb 37.1 strongly depleted normal B-cells as well as spiked B-lymphoma cells in blood samples from healthy donors as well as malignant B-cells in blood from CLL patients. In all assays, mAb 37.1 was superior to rituximab in terms of potency and maximal cell lysis. A single dose of mAb CD37.1 administered to human CD37-transgenic mice resulted in a reversible, dose-dependent reduction of peripheral B-cells. In a Ramos mouse model of human B-cell lymphoma, administration of mAb 37.1 strongly suppressed tumor growth. Finally, a surrogate Fc-engineered antibody to macaque CD37, with in vitro pro-apoptotic and ADCC activities very similar to those of mAb 37.1, induced dose-dependent, reversible B-cell depletion in cynomolgus monkeys. In conclusion, the remarkable preclinical pharmacodynamic and antitumor effects of mAb 37.1 warrant clinical development for B-cell malignancies.
Introduction

Chemo-immunotherapy incorporating the CD20-specific monoclonal antibody (mAb) rituximab has emerged as standard clinical practice for treatment of B-cell non-Hodgkin’s lymphoma (NHL)\textsuperscript{1,2,3} and chronic lymphocytic leukemia (CLL).\textsuperscript{4,5} However, a significant portion of patients eventually relapse after rituximab treatment, creating a need for alternative approaches. A fully human CD20 antibody, ofatumumab, which was recently approved for the treatment of CLL patients who are refractory to fludarabine and alemtuzumab, is under active investigation as first line therapy in combination with chemotherapy.\textsuperscript{6}

While rituximab displays clinically relevant antitumor activity in combination with chemotherapy, direct pro-apoptotic and antibody-dependent cell-mediated cytotoxicity (ADCC) mediated by rituximab is moderate.\textsuperscript{7} Work with the first generation of mAbs including rituximab has provided valuable insights into how to engineer novel mAbs with high antitumor efficacy. Advances in our understanding of the interactions of the antibodies with their receptors on immune effector cells provide a rationale for generating engineered antibodies with high affinity to Fcγ receptors that translates into more potent ADCC. In particular, clinical response to rituximab has been correlated with the presence of certain allelic variants of FcγR exhibiting increased binding to the antibody.\textsuperscript{8,9} To date, several mAbs optimized for FcγR affinity have been described, including mAbs directed against CD20 (GA101,\textsuperscript{7} LFB-R603,\textsuperscript{10} AME-D\textsuperscript{11}); in particular, the CD20 mAb GA101, shows enhanced ADCC relative to rituximab and is under investigation for therapy of B-cell leukemias and lymphomas.\textsuperscript{7}

As an alternative to the prototypic B-cell target CD20, a variety of other leukemia and lymphoma cell-surface antigens have been investigated, including CD19, CD22,
CD23, CD37, CD40, and CD80. The tetraspanin CD37 is predominantly expressed on mature B-cells, with highest expression levels on peripheral blood B-cells, and lower levels on plasma cells. Low-level expression has been reported on T-cells, granulocytes and monocytes, while CD10+ precursor cells are CD37-negative.12-15 Importantly, strong and homogeneous CD37 expression has been detected on the surface of B-cell leukemia and lymphoma cells.16-20 This favorable expression profile led to the exploration of CD37-directed radioimmunotherapy in patients suffering from B-cell lymphoma using the murine CD37 mAb MB-1.21,22 A CD37-specific small modular immunopharmaceutical (SMIP), an IgG1-like single-chain antibody with a truncated constant region, is in early clinical development for CLL.23

We report here on the pharmacological profile of a novel mAb against human CD37 that we have generated and engineered for improved effector function. mAb 37.1 is an Fc-engineered mouse/human chimeric IgG1 antibody in which two amino acids have been replaced in the Fc region in order to increase affinity to FcγRIIIa.
Methods

Antibodies

All monoclonal IgG1 antibodies (mAbs) against human CD37 used in this study were expressed in DHFR-deficient Chinese hamster ovary (CHO) DG44 suspension cells under serum-free conditions and purified via MabSelect Protein A affinity chromatography (GE Healthcare). MAb 37.1-wt is a mouse–human chimeric IgG1 antibody derived from the murine mAb G28.1. Fc-variants of mAb 37.1-wt were constructed by introducing amino acid substitutions in the Fc CH2-domain of the wildtype IgG1 via quick change mutagenesis.24 A surrogate IgG1 mAb 37_surr antibody was generated by immunization of mice with cynomolgus monkey CD37-expressing recombinant cells and chimerized and Fc-engineered as for the anti-human CD37 mAbs. Clinical grade rituximab was obtained from Roche (Basel, Switzerland); clinical grade alemtuzumab was obtained from Berlex (Montville, NJ).

Fcγ receptors

Ig-like FcγRIIIa (CD16a; allelic variants 158V and 158F) and FcγRI (CD64) were expressed in DHFR-deficient Chinese hamster ovary (CHO) DG44 suspension cells under serum-free conditions and subsequently purified via IgG Sepharose 6 FF affinity chromatography (GE Healthcare).

Cell lines, donors, and patients

Ramos Burkitt lymphoma cells were obtained from ATCC (CRL-1596). Master cell banks and working cell banks were established according to Boehringer Ingelheim Regional Centre Vienna standards. Blood samples from healthy volunteers were obtained from the Austrian Red Cross. Patient samples were provided by the University of Ulm, Germany, and were
obtained after informed consent in accordance with the Declaration of Helsinki and with approval of the UK Home Office and the cantonal ethical committee in Austria.

**FACS Scatchard analysis**

The affinity of the antibody to cellular antigen was calculated by Scatchard analysis using a FACS based method for quantification of bound antibody, using CD37-positive Ramos Burkitt lymphoma as target cell, as detailed in the online supplement provided with this manuscript.

**Surface plasmon resonance (SPR)**

All kinetic interaction experiments between CD37 mAbs and FcγRs CD16 (allelic variants V158 and F158) and CD64, respectively, were performed at 25°C using a Biacore T200 instrument (GE Healthcare), as detailed in the online supplement.

**Apoptosis assay**

Apoptosis activity was determined in Ramos Burkitt lymphoma cells and primary CLL cells after 24-hour incubation with mAbs in the presence or absence of an IgG cross-linking antibody (goat anti-human IgG, Sigma I3382) by Annexin V staining. Assays were performed in cultured cells in triplicate, and in CLL blood samples from six individual donors, as detailed in the online supplement. The degree of apoptosis induction is displayed as the total percentage of AnnexinV positive cells corrected for spontaneous lysis.

**ADCC Assay**

ADCC assays with cultured Ramos cells or primary CLL cells were performed by 3-hour co-cultivation of target (T) cells and interleukin-2 stimulated effector (E) peripheral blood mononuclear cells from healthy volunteers (E:T ratio, 25:1).
Cytotoxicity was determined by measurement of released lactate dehydrogenase (LDH) and the percentage of specific cell lysis was calculated according the formula
\[(\text{effector/target cell mix – effector cell control – spontaneous release}) / (\text{maximal release – spontaneous release})\]. The co-cultivation of effector cells with target cells in presence of antibody was performed as detailed in the online supplement.

**In vivo pharmacokinetic and pharmacodynamic studies in CD37 transgenic mice and cynomolgus monkey**

Pharmacodynamic (PD) effects in peripheral blood cells were assessed in HuCD37-transgenic mice, and pharmacokinetic (PK)/PD effects were assessed in peripheral blood of cynomolgus monkeys. Mouse strains and care are fully detailed in the online supplement. A single injection of human CD37 mAb (at doses of 0.0025/0.025/0.25/2.5/25 mg/kg) was administered into the lateral tail vein of female HuCD37-transgenic (murine CD37 knock-out [k.o.]; human CD37 knock-in [k.i.]) mice (3 animals per group). Vehicle-treated animals served as a control group. Peripheral blood cell counts were determined one day before dosing and on days 2, 7, 15, and 22 thereafter. B-cell counts were determined by FACS analysis of B220+ lymphocytes. B-cell counts were normalized to control values at the same day and the degree of B-cell depletion relative to control was calculated for each animal.

Cynomolgus monkeys (*Macaca fascicularis*) received a single dose of mAb 37_surr via tail-vein infusion at an infusion rate of approximately 0.5 mL/min over a 30-minute period. Blood samples were collected from the femoral vein and transferred into tubes containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant. Blood samples were assayed by flow cytometry using the following marker combinations: CD20/CD21/CD27/CD40 for B-cells and CD3/CD4/CD8/CD25 for T-cells. B-cell counts were normalized to baseline values on day -7 (7 days before start of
and the degree of B-cell and T-cell depletion relative to baseline was calculated for each animal. For PK analysis of mAb CD37\textsubscript{surr}, cynomolgus blood samples were obtained from each animal for plasma concentrations at pre-dose, 0.5, 2, 8 and 24 h after dosing and then on days 4, 8, 15, 22, 29 and 36. A sandwich ELISA was used to quantify total human IgG using a polyclonal anti-human IgG (H&L chain) antibody non-cross-reactive with monkey IgG. Plasma concentration-time data were analyzed by non-compartmental methods with the programme ToxKin 3 using individual plasma concentration-time curves.

**In vivo antitumor efficacy studies**

To establish subcutaneous tumors in mice, Ramos cells were harvested by centrifugation, washed and resuspended in PBS + 5% FCS at 5 x 10\textsuperscript{7} cells/mL. 100 µL cell suspension containing 5 x 10\textsuperscript{6} cells was then injected subcutaneously into the right flank of the mice (1 site per mouse). Mice were randomly distributed between the treatment and the vehicle control group (15 days after cell injection) when tumors were well established and had reached diameters of 6 to 8 mm. Antibodies were administered intravenously into the tail vein in an administration volume of 100 µL per mouse every 3rd or 4th day. The tumor diameter was measured three times a week (Monday, Wednesday and Friday) with a caliper. The volume of each tumor [in mm\textsuperscript{3}] was calculated according to the formula “tumor volume = length*diameter\textsuperscript{2} \pi /6”. To monitor side effects of treatment, mice were inspected daily for abnormalities and body weight was determined three times a week. Animals were sacrificed at the end of the study when the control tumors reached a median size of approximately 1500 mm\textsuperscript{3}.

**Statistical analysis**
In PD studies of mAb 37.1 in mice, an analysis of covariance (ANCOVA) with baseline values (Day -1) as covariate and the factor dose group (vehicle control, and 0.0025, 0.025, 0.25, 2.5, and 25 mg/kg) was performed for each time point (day 2, 7, 15, and 22). The error term of the ANCOVA was taken as estimate for the variability in the following t-tests, comparing the dose groups vs. vehicle control. For the adjustment of the respective p-values for multiple testing a procedure for ordered alternatives was used. Assuming an increasing effect with increasing dose, the highest dose was tested first and treatment at next lowest dose was only implemented if a statistically significant difference versus the vehicle control group was demonstrated. A p-value of less than 0.05 was considered to show a difference between the dose groups and vehicle control. In PD studies in the monkey, an analysis of variance (ANOVA) for repeated measurements with fixed factors dose group and time point was performed.
Results

Binding to CD37

Binding to cellular antigen was determined by FACS Scatchard analysis using Ramos Burkitt lymphoma cells as the target. mAb 37.1 bound surface expressed CD37 with an apparent affinity of 1.3 nM (mean, 3 experiments). The antibody bound to recombinant CHO cells expressing human CD37 with similar affinity, but did not bind to the parental CHO cells (data not shown).

Binding to Fcγ Receptors

Binding parameters of CD37 mAbs interacting with Fcγ receptors CD16-158V, CD16-158F, and CD64 were determined by performing kinetic SPR experiments as detailed in the supplement. Data from one representative experiment out of three are summarized in Table 1 and in the supplement table S1. Non-Fc-engineered mAb 37.1-wt bound CD16-158V and CD16-158F with affinities (KD1) of 215 nM and 1,205 nM, respectively (Table 1; Figure S2A, C; Table S1). Our Fc-engineered mAb 37.1 displayed a more than 50-fold increase in affinity to CD16-158V (KD1 = 4 nM) and a more than 40-fold increase in affinity to CD16-158F (KD1 = 28 nM), respectively (Table 1; Figure S2B, D; Table S1). In addition, we analyzed binding of both mAbs to CD64 and observed an 8-fold increase in affinity of our Fc-engineered mAb 37.1 (KD = 20 pM) when compared to mAb 37.1-wt (KD = 164 pM) (Table 1; Figure S2E, F; Table S1).

Proapoptotic activity

Direct apoptosis induction in the absence of complement factors and effector cells was assessed on Ramos Burkitt lymphoma cells. Results of Annexin V staining after 24 hours exposure showed that mAb 37.1 exerted potent pro-apoptotic activity without IgG cross-linking, which was further increased by cross-linking with anti-IgG.
(representative examples from 3 independent assays for each condition are shown in Figure 1A and 1B). The proportions of Annexin V-positive cells induced by mAb 37.1 were 37% (range 30-43%) in the absence and 46% (range 39-52%) in the presence of a cross-linking antibody. Apoptosis induction was concentration dependent with EC$_{50}$ values in the range of 40-100 ng/mL.

Compared to rituximab, mAb 37.1 displayed significant (p < 0.05) higher induction of Ramos cell apoptosis at a saturating mAb concentration of 100 µg/mL, with or without cross-linking (mean of four assays is shown in Figure 1C). Importantly, Ramos cells express similar levels of CD20 and CD37 as determined by FACS Scatchard analysis (approximately 110,000 molecules/cell; data not shown). Time-lapse light microscopy of Ramos cells showed that mAb 37.1 induced rapid cell clustering in the absence of IgG cross-linking, with initial signs of clustering already 1 hour after addition of mAb 37.1 and maximal aggregation after 3 hours of incubation (Figure 2). In contrast, rituximab-treated cells showed similar behavior as buffer controls (Figure 2).

To assess whether apoptosis could also be induced in primary, patient-derived CLL cells, PBMC preparations from 4-six patients were tested at saturating antibody concentration of 30 µg/mL. Similar to effects on Ramos cells, pro-apoptotic activity seen with mAb 37.1 was significantly higher than with rituximab both in the absence and presence of an IgG cross-linking antibody (p < 0.05; Figure 1D). As described above for Ramos cells, rapid cell clustering induced by mAb 37.1 was observed in blood samples from CLL patients (Figure 2B).
ADCC

To investigate cell-mediated cytotoxicity, ADCC assays using Ramos target cells and IL-2-stimulated PBMC from healthy donors as effector cells were performed. mAb 37.1 showed strongly enhanced potency and efficacy of cytolysis (Figure 3A) in comparison to the wild-type (non-Fc-engineered) parental antibody. The average EC$_{50}$ value was 2 ng/mL for mAb 37.1 (mean 3 assays). Maximal cell lysis at saturating antibody concentrations varied from 29% to 71% dependent on donor PBMC. Direct comparison to rituximab revealed clearly improved maximal cell lysis and potency when assessed side by side using the same effector cell preparations (a representative example is shown in Figure 3B).

In addition, the ADCC activity of mAb 37.1 on primary CLL mononuclear cells from peripheral blood of 6 individual patients was assessed. MAb 37.1 was superior to rituximab with 2 to 3-fold higher maximal cell lysis and about 100-fold improved potency (Figure 3C and 3D).

Whole blood assays

The effect of antibodies on normal B-cells was assessed in autologous whole blood depletion assays using blood samples from healthy donors. Whereas rituximab displayed only marginal activity in this assay type, mAb 37.1 demonstrated high efficacy in the range of 50% to 60% depletion in an initial set of three different donors (Figure 4A). The effect on Ramos lymphoma cells spiked into the blood samples was assessed in parallel in these 3 samples. The depleting effect of mAb 37.1 was comparable to that on B-cells (mean of 57%, Figure 4B) whereas rituximab showed only marginal activity. To further substantiate the effects observed with mAb 37.1, seven additional blood samples from healthy individuals were investigated. B-cell depletion at antibody concentrations from 3 µg/ml to 300 µg/ml ranged from 39% to
70% (mean: 53%) and thus confirmed efficient B-cell depleting activity of mAb 37.1. Potential effects on other leukocyte subsets were assessed in parallel by enumeration of CD3 positive T-cells, CD14 positive monocytes, and granulocytes before and after incubation with mAb 37.1. No depleting activity for mAb 37.1 on these cell types was observed, whereas alemtuzumab, a mAb specific for CD52, displayed T-cell depleting activity (not shown).

Superior depletion compared to rituximab was also seen in peripheral blood cells obtained from a cohort of patients with CLL (n=8). Incubation with mAb 37.1 for 3h or 8h led to a concentration-dependent B-cell depletion that was superior to rituximab-induced depletion (Figure 4A and 4D). The mean maximum depletion of about 80% at a mAb concentration of 10 µg/ml was already achieved after 3h incubation (Figure 4C).

**In vivo studies**

**Pharmacodynamic effect on B-cells in HuCD37 transgenic mice**

mAb 37.1 lacks cross-reactivity to murine CD37 (data not shown). A transgenic murine CD37 k.o. / human CD37 k.i. mouse was therefore generated as a tool to assess the in vivo pharmacodynamic properties of CD37 mAbs. Analysis of isolated transgenic splenocytes demonstrated expression of human CD37 at a density of about 10,000-12,000 sites per cell, and the binding affinity of mAb 37.1 was approximately 0.3 nM and thus comparable to that for human B-cells (data not shown).

In transgenic mice, a single injection of mAb 37.1 showed dose-dependent reduction of peripheral B-cells, with almost complete depletion at the 2.5 and 25 mg/kg dose levels (Figure 5A). The B-cell nadir occurred between days 2 and 7 after dosing. B-
cell reduction in the 25 and 2.5 mg/kg groups was statistically significant on all days during the study (p<0.001 for nadir values). At the end of the study (day 22), B-cell counts reached approximately 50% and 75% of predose values in the 25 and 2.5 mg/kg groups, respectively, indicating that depletion is reversible. Dose-response analysis on day 2 yielded an ED$_{50}$ of 422 µg/kg (Figure 5B). B-cell depletion was accompanied by a transient reduction of T-cells, granulocytes, monocytes, and platelets, but not of red blood cells (not shown).

**Pharmacodynamic effects of a surrogate mAb in cynomolgus monkeys**

As mAb 37.1 lacks cross-reactivity with CD37 orthologues from all monkey species tested, we have developed cynomolgus-specific chimeric, Fc-engineered surrogate mAb, mAb 37$_{surr}$, with comparable binding and *in vitro* functional properties as mAb 37.1. This antibody demonstrated potent ADCC activity against recombinant HEK293 cells expressing cynomolgus CD37, potent pro-apoptotic activity in the presence and absence of IgG cross-linking, and depletion of naïve B-cells but not T-cells in cynomolgus blood *in vitro*. Analysis of cynomolgus blood cells revealed similar binding to B- and T-cells as mAb 37.1 in humans (Table 2). These data validate mAb 37$_{surr}$ as a relevant surrogate antibody for mAb 37.1 to assess CD37-specific antibody effects in the cynomolgus monkey.

The effect of a single infusion of mAb 37$_{surr}$ (doses 10 mg/kg, 0.3 mg/kg, 0.01 mg/kg) on peripheral blood B- and T-cells was assessed by flow cytometry. At 24-hr post infusion, B-cell numbers decreased sharply in the two high-dose animals (from 1213 to 75 cells/µL and from 1596 to 253 cells/µL) (Figure 5C). The effect was attenuated in the medium-dose group (295 and 713 cells/µL). No effect was seen in the animals treated with the lowest dose of mAb 37$_{surr}$. At day 8, no significant effect was seen for the medium- and low-dose animals whereas the B-cell numbers had further declined in the two animals treated with the high dose of mAb 37$_{surr}$ (6 and 19 cells/µL).
Subsequently the number of B-cells increased in these animals and reached about 30% of predose values on day 36 (end of study). B-cell reduction was statistically significant for the high dose at all time points after infusion (p<0.0001).

A decrease of T-cells, neutrophils, monocytes and platelets was observed in animals treated with the high dose of mAb 37\textsubscript{surr} only. These effects were transient with cell nadirs occurring between days 1 and 8 of the study. The maximum reduction varied between 40% (platelets) and 70% (T-cells), effects were not statistically significant. No effect on red blood cells was observed.

PK evaluation revealed that the exposure to mAb 37\textsubscript{surr} was substantial in the intermediate and high dose group and AUC (0-840h) values increased considerably more than proportionally with dose (see Table 3). In the low dose group (0.01 mg/kg), all assay results were below the lower limit of quantification (60 ng/mL). In the intermediate dose group (0.3 mg/kg), PK evaluation was restricted as plasma concentrations were measurable only until 8 h after administration, and the calculated half-life (6h) was not considered as valid (Table 3). The terminal half-life was about 60 h in the high dose group (10 mg/kg).

**Antitumor effect in a Ramos xenograft mouse model**

To assess the antitumor efficacy of mAb 37.1 in comparison to rituximab, Ramos cells were injected subcutaneously into the flank of immunodeficient “nude” mice, and mAb treatment at doses of 25 mg/kg given twice weekly i.v. was initiated when the tumors had reached a median volume of about 60 mm\(^3\) (Figure 6). Both antibodies caused significant growth retardation compared to vehicle treatment (day 15); the difference between mAb 37.1 and rituximab was not statistically significant.
Discussion

The use of mAbs to achieve B-cell depletion has been an important advance in treatment of lymphoid malignancies, and the CD20 mAb, rituximab has been incorporated into standard of care for both NHL and CLL. With increasing exposure of patient populations to rituximab, the frequency of resistance to the agent is on the rise, for example by emergence of malignant clonal cells with loss of CD20 from the cell surface.\textsuperscript{25} In addition, rituximab has limited activity in depletion of CLL cells as monotherapy, which is held to be related to the lower density of CD20 on these cells and to the low efficacy of direct rituximab cytotoxicity.\textsuperscript{26} Recent data from the glyco-engineered CD20 mAb GA101 demonstrated that both an increase in direct cytotoxicity and improved effector-cell-mediated cytotoxicity can yield superior preclinical activity against malignant B-cells.\textsuperscript{7} Several clinical trials are underway (http://clinicaltrials.gov) to investigate if such preclinical superiority will lead to improved benefit for cancer patients.

Several other cell-surface receptors on malignant B-cells have been the subject of intense research within the past decade.\textsuperscript{27} Among those, CD37 is a promising target in B-cell malignancies due to its high expression in many lymphoid cancers and on CLL cells.\textsuperscript{13,20} A recently developed CD37 IgG-like molecule directed against CD37 exerts pro-apoptotic and ADCC activity in preclinical settings and showed signs of clinical activity in a phase I trial in patients with relapsed, refractory CLL.\textsuperscript{28,29}

Our novel CD37 mAb 37.1 was engineered within the Fc region of the parent IgG1 molecule for increased binding affinity to FcγRIIIa, as has been described for other mAbs.\textsuperscript{24} Our binding data confirm strongly enhanced binding affinity of the Fc-engineered mAb 37.1 to FcγRIIIa (both CD16-158F and CD16-158V) to a similar extent as reported for CD19 mAbs.\textsuperscript{30} Interestingly, also the binding affinity to FcγRI

\textsuperscript{13}
(CD64) was enhanced about 8-fold in case of mAb 37.1, whereas Horton and co-workers published only marginally increased binding of their Fc-engineered CD19 mAb to CD64.\textsuperscript{30}

mAb 37.1 shows strongly enhanced cytotoxicity compared with the parent IgG1 molecule on Ramos Burkitt lymphoma cells and CLL cells from patients when tested in a heterologous ADCC assay. Importantly, our CD37 mAb also showed clearly improved ADCC activity when tested side by side with rituximab on Ramos cells, which show similar levels of CD20 and CD37 antigen density. Enhancement of ADCC appeared to be more even pronounced on primary CLL cells than on Ramos cells, which may be explained by a higher number of CD37 molecules than of CD20 on these cells (K.-H. Heider, unpublished observation). In summary, the enhancement of Fcy receptor binding in this novel CD37 mAb leads to clearly improved ADCC compared to rituximab both on lymphoma cells with similar antigen densities for CD20 and CD37 and on primary CLL cells.

In addition to improved ADCC function, mAb 37.1 was selected for its potential to exert strong and potent direct cell killing. The pro-apoptotic activity against Ramos cells and primary CLL cells was clearly superior compared to rituximab. mAb 37.1 induces significant apoptosis without IgG cross-linking on Ramos cells and primary CLL cells, which is significantly superior to that of Rituximab, which induces only marginal pro-apoptotic activity in both cell types. After cross-linking, the pro-apoptotic activity of both mAbs increased, however less pronounced on primary CLL cells than on Ramos cells. This finding may be explained by a higher heterogeneity of primary CLL cells compared to Ramos cells, differences in levels of antigen expression, and a considerably higher level of spontaneous apoptosis in primary cells than in Ramos cells. Apoptotic cell killing by mAb 37.1 does not require IgG cross-linking and thus the mode of direct cell death induction induced by mAb 37.1 resembles that of so-
called type II CD20 antibodies like tositumomab and GA101. Type II CD20 mAbs are characterized by reduced ability to mediate CDC activity compared to type I antibodies, but have been demonstrated to outperform type I antibodies with respect to their B-cell depleting activity.\textsuperscript{7,31} In this context it is interesting to note that mAb 37.1 likewise lacks CDC activity (data not shown). Furthermore, the observed phenotypic changes on Ramos cells and primary CLL cells after incubation with mAb 37.1 (e.g. rapid homotypic aggregation in the absence of a cross-linking mAb) provide evidence that mAb 37.1 can be considered to be a type II CD37 antibody. Homotypic aggregation is a characteristic of type II antibodies and is associated with highly effective direct cell killing.\textsuperscript{32} Additional studies are needed to further elucidate the mode of direct cell killing induced by mAb 37.1, e.g. whether a redistribution of target molecules in lipid rafts occurs.

Both ADCC and apoptosis are considered relevant for the clinical activity of B-cell directed antibodies in hematological malignances.\textsuperscript{33,34} However, the relevance of \textit{in vitro} assays for prediction of responses \textit{in vivo} appears to be limited due to the artificial nature of the test systems (e.g. heterologous effector cells, lack of endogenous IgG levels). To compensate for these limitations we used whole blood assays, which are considered to mimic the \textit{in vivo} situation more closely due to the presence of autologous effector cells and IgG levels, and furthermore are able to reflect the interplay of several modes of action with respect to the depleting activity of the antibody. Results from whole blood assays, using quantitative FACS methodology, showed that the CD37 mAb described here is approximately 10-fold more effective in depleting autologous B-cells, Ramos lymphoma cell-spiked samples, and primary CLL cells than rituximab. Although a direct comparison with the third-generation, glyco-engineered CD20 mAb GA101 has not been possible to date, the results of whole blood assays appear to compare favorably with recently
published data on GA101.\textsuperscript{7,26} Taken together, our data and literature data suggest that targeting CD37 on the surface of malignant CLL cells may result in a higher degree of cell depletion than targeting CD20, which ultimately may translate into better clinical efficacy.

No effects on cells with low levels of antigen (T-cells, monocytes, granulocytes) were observed in the whole blood assay using blood from healthy individuals, whereas an antibody known to have T-cell depleting activity (alemtuzumab) showed significant effects in these assays (data not shown). Thus, the level of target antigen expression appears to be below a threshold required for antibody induced cytotoxic effects, at least in the applied \textit{in vitro} test system.

In order to assess the \textit{in vivo} pharmacodynamic activity of our CD37 mAb, two different approaches were used. Due to the lack of cross-reactivity of mAb 37.1 with CD37 orthologues of any animal species tested, a transgenic mouse model was established, in which the murine CD37 gene was replaced by human CD37. In addition, a surrogate antibody reactive with cynomolgus CD37 was developed in order to allow \textit{in vivo} assessments in an animal species with a FcγR system close to humans.\textsuperscript{35}

A pharmacodynamic study in huCD37 transgenic mice with mAb 37.1 showed that complete B-cell depletion in peripheral blood could be achieved at a single dose of 2.5 mg/kg, with nadirs at days 2-7 and recovery to at least 50% of pretreatment levels within 3 weeks. In the cynomolgus monkey, a single, intermediate dose of 0.3 mg/kg surrogate mAb CD37\textsubscript{surr} lead to a significant reduction in peripheral B-cells, which was complete and long-lasting at the 10 mg/kg dose level. The recovery of peripheral blood B-cells indicated that at the doses applied no substantial effects on bone marrow precursor cells were imposed. In both species, a transient reduction of
T-cells was observed which paralleled that of B-cells. In the mouse, these effects were similar to that on B-cells, whereas in the monkey a T-cell reducing effect was observed at the 10 mg/kg dose level only, and was far less pronounced than that observed on B-cells. The effect on T-cells may be attributed to the low level of CD37 expression on T-cells. It is currently unknown why the effects on transgenic murine T-cells were more pronounced than those on cynomolgus T-cells. Antigen expression levels on T-cells were similar in both species and hence do not explain the substantial difference observed in vivo.

It has to be noted that a transient reduction of peripheral T-cells has also been observed in clinical trials with CD20 antibodies where the CD20 antigen is not expressed on T-cells. Hence, a transient effect on T-cells may also be caused by lymphocyte redistribution effects rather than by cell killing. In our in vitro whole blood assay, we did not detect any T-cell reducing effect of mAb 37.1 nor mAb CD37sur. Together, our data indicate that depletion of CD37-expressing cells is only transient on non-malignant peripheral leukocytes, suggesting an acceptable safety profile of these antibodies, and supports further investigation in clinical trials in cancer patients.

Targeting CD37 using mAb 37.1 substantially limited the growth of established Burkitt lymphoma in a Ramos xenograft model. The effect was comparable to that achieved with rituximab. However, mice are not considered to be a suitable species with respect to the cytotoxicity-enhancing effect of Fc-mutations. Hence, the ADCC-mediated antitumor effects of mAb 37.1 may be underestimated in the mouse model used in this study.

In conclusion, our data indicate that the novel, Fc-engineered mAb directed against human CD37 described in this paper has substantially greater B-cell depleting activity in several in vitro systems than rituximab, and displays profound
pharmacodynamic and anti-tumor effects \textit{in vivo}. This antibody is a promising candidate for treatment of patients with B-cell malignancies and warrants further clinical evaluation.
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Authorship and Conflict of Interest Statements

Karl-Heinz Heider, Kerstin Kiefer, Elinborg Ostermann, Anke Baum, Herbert Lamche, Zaruhi Küpcü, Alexander Jacobi, Steffen Müller, Ulrich Hirt, Günther Adolf, and Eric Borges are employees of Boehringer Ingelheim. Thorsten Zenz: Honoraria, research funding; Matthias Volden: none; Stephan Stilgenbauer: Honoraria, research funding.

KHH, SS, GRA and EB initiated and supervised studies. ZK established assays, KHH, KK, EO, AB, HL, ZK, AJ, SM, UH, TZ, MV performed experiments and analyzed data. KHH wrote the paper. GRA, AB, SM, UH, TZ, SS and EB edited the paper.
References


Tables

Table 1. Biacore analysis of mAb 37.1 and mAb 37.1-wt binding to Fcγ-receptors.

Results from one representative experiment of 3 are shown.

<table>
<thead>
<tr>
<th>Fcγ receptor</th>
<th>Dissociation constant</th>
<th>37.1-wt</th>
<th>37.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD16 158V</td>
<td>KD1 (nM)</td>
<td>215</td>
<td>4</td>
</tr>
<tr>
<td>CD16 158 F</td>
<td>KD1 (nM)</td>
<td>1205</td>
<td>28</td>
</tr>
<tr>
<td>CD64</td>
<td>KD1 (nM)</td>
<td>0.16</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Table 2. Characteristics of mAb 37.1 and mAb 37surr

<table>
<thead>
<tr>
<th>Function</th>
<th>Cell type (Human/cynomolgus)</th>
<th>mAb 37.1</th>
<th>mAb 37surr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apparent affinity (KD)</td>
<td>B-cells/B-cells</td>
<td>0.4 nM</td>
<td>0.2 nM</td>
</tr>
<tr>
<td></td>
<td>T-cells/T-cells</td>
<td>1.7 nM</td>
<td>1.4 nM</td>
</tr>
<tr>
<td>Antigen density (sites/cell)</td>
<td>B-cells/B-cells</td>
<td>53000</td>
<td>55000</td>
</tr>
<tr>
<td></td>
<td>T-cells/T-cells</td>
<td>1900</td>
<td>1900</td>
</tr>
<tr>
<td>ADCC (EC50)</td>
<td>Ramos/macCD37-CHO</td>
<td>0.01 nM</td>
<td>0.02 nM</td>
</tr>
<tr>
<td></td>
<td>(2 ng/mL)</td>
<td>(4 ng/mL)</td>
<td></td>
</tr>
<tr>
<td>Apoptosis (w/o x-link) (EC50)</td>
<td>Ramos/macCD37-CHO</td>
<td>0.4 nM</td>
<td>9.3 nM</td>
</tr>
<tr>
<td></td>
<td>(66 ng/mL)</td>
<td>(1423 ng/mL)</td>
<td></td>
</tr>
<tr>
<td>Apoptosis (with x-link) (EC50)</td>
<td>Ramos/macCD37-CHO</td>
<td>0.3 nM</td>
<td>10 nM</td>
</tr>
<tr>
<td></td>
<td>(41 ng/mL)</td>
<td>(1574 ng/mL)</td>
<td></td>
</tr>
<tr>
<td>CDC</td>
<td>Ramos/macCD37-CHO</td>
<td>negative</td>
<td>negative</td>
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<tr>
<td>Whole blood (EC50)</td>
<td>B-cells/B-cells</td>
<td>0.2 nM</td>
<td>0.9 nM</td>
</tr>
<tr>
<td></td>
<td>(33 ng/mL)</td>
<td>(140 ng/mL)</td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Pharmacokinetic parameters of mAb 37\textsubscript{surr} in cynomolgus monkey

<table>
<thead>
<tr>
<th>Parameter</th>
<th>0.01 mg/kg</th>
<th>0.3 mg/kg</th>
<th>10 mg/kg</th>
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</thead>
<tbody>
<tr>
<td>C(max) [µg/mL]/[mg/kg]</td>
<td>NC</td>
<td>24.9</td>
<td>24.1</td>
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<tr>
<td>AUC(0-804h) [µg⋅h/mL]/[mg/kg]</td>
<td>NC</td>
<td>140</td>
<td>1720</td>
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<tr>
<td>t(1/2) [h]</td>
<td>NC</td>
<td>5.7</td>
<td>~60</td>
</tr>
</tbody>
</table>
Figures and Figure Legends

Legend to Figure 1. Apoptosis assays.

Apoptosis induction (% total Annexin V positivity) of Ramos lymphoma cells after 24-hour incubation with: (A) Increasing concentrations of mAb 37.1 in the absence of an IgG cross-linking antibody; (B) Increasing concentrations of mAb 37.1 in the presence of an IgG cross-linking antibody; and (C) 100 µg/mL mAb 37.1 or rituximab in the absence (-) or presence (+) of an IgG cross-linking antibody. Bars represent mean of four experiments, SD is indicated. Panel D shows apoptosis induction (% total Annexin V positivity above spontaneous apoptosis) of primary CLL cells from 6 donors after 24-hour incubation with 30 µg/mL mAb 37.1, or rituximab in the absence or presence of an IgG cross-linking antibody. Bars represent mean of six donors, SD is indicated.

Legend to Figure 2. Phenotypic analysis.

Phenotypic analysis of antibody treatment on Ramos cells (A) and primary CLL cells (B). Ramos cells were incubated with mAb 37.1 or rituximab at a concentration of 30 µg/mL and photomicrographs were taken at the indicated time points using an automated camera system. Blood samples from CLL patients were incubated with mAb 37.1 and rituximab for 1 h and blood smears were analyzed after routine staining (Giemsa).

Legend to Figure 3. Antibody-dependent cell-mediated cytotoxicity

ADCC activity (% cytolysis) on Ramos cells (E:T ratio 25:1) of mAb 37.1 in comparison to non-Fc-engineered parental mAb (37.1-wt) (A) and rituximab (B). ADCC activity on primary CLL cells of 30 µg/mL of mAb 37.1 and rituximab showing (C) potency (EC50 in ng/mL) and (D) efficacy (maximal cell lysis in %). Bars represent mean of 6 donors, SD is indicated.
Legend to Figure 4. B-cell depletion of whole blood samples.
Depletion (in %) of autologous B-cells (A) and spiked Ramos cells (B) assessed by quantitative FACS analysis (TruCount) of whole blood samples from normal individuals after 3-h incubation with 30 μg/mL of mAb 37.1 and rituximab. Bars represent mean of 3 donors, SD is indicated. In whole blood assays with primary CLL samples mAb 37.1 shows concentration-dependent CLL cell depletion whereas rituximab displays only marginal activity after 3 h (C) and 8 h (D) incubation. Bars represent mean of 8 donors, SD is indicated.

Legend to Figure 5. Dose response in animals.
Effect of a single dose of mAb 37.1 (A) on peripheral B-cell count (B220+ cells) in huCD37 transgenic mice. Each curve represents the mean of 3 animals per dose group. B) Dose response of B-cells on day 2 after treatment; Absolute B-cell counts for mAb 37.1 are shown; mean of 3 animals, SD is indicated. C) Effect of a single dose of mAb 37\textsubscript{surr} on peripheral blood B-cell count in cynomolgus monkeys. Relative B-cell counts compared to pre-dose (day -7) are depicted. Mean 2 animals per dose group.

Legend to Figure 6. Effect on tumor volume.
Effect of antibody treatment on growth of established Ramos sc tumors in NMRI mice. Ten mice per cohort were treated twice weekly with mAb 37.1 and rituximab at 25 mg/kg for a total of 2 weeks. Median tumor volume is shown.
Figure 2

A

1 hour  3 hours  12 hours  24 hours

100 μm

PBS

mAb 37.1

Rituximab
Figure 3
Figure 4

A  B Cells  B  Ramos

% Depletion

mAb 37.1  Rituximab

% Depletion

mAb 37.1  Rituximab
% CD19+ cells normalized to negative control

Concentration

100 µg/ml 10 µg/ml 1 µg/ml 100 µg/ml 10 µg/ml 1 µg/ml

8h

mAb 37.1

Rituximab
Figure 5

A

CD37 10/08 B Cells

- Vehicle
- 25 mg/kg
- 2.5 mg/kg
- 0.25 mg/kg
- 0.025 mg/kg
- 0.0025 mg/kg

![Graph showing CD37 10/08 B Cells over time and different doses.]

B

Log antibody dose (µg/kg)

![Graph showing log antibody dose with mAb 37.1 data.]

C

515655 FACS Blood

- 0.01 mg/kg
- 0.3 mg/kg
- 10 mg/kg

![Graph showing 515655 FACS Blood over time and different doses.]

B cells: relative cell count (%)
Figure 6

![Graph showing the median tumor volume in mm$^3$ over days for control, mAb 37.1, and Rituximab treatments.](image)
A novel Fc-engineered monoclonal antibody to CD37 with enhanced ADCC and high pro-apoptotic activity for treatment of B-cell malignancies