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High CD33-antigen loads in peripheral blood limit the efficacy of gemtuzumab ozogamicin (Mylotarg®) treatment in acute myeloid leukemia patients

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Gemtuzumab ozogamicin (Mylotarg®) induces remission in approximately 30% of relapsed AML patients. We previously demonstrated that gemtuzumab infusion results in near-complete CD33 saturation in peripheral blood, and that saturating gemtuzumab levels result in continuous binding and internalization of gemtuzumab due to renewed CD33 expression. We now demonstrate that a high CD33-antigen load in peripheral blood is an independent adverse prognostic factor, likely due to peripheral consumption of gemtuzumab. Indeed, CD33 saturation in bone marrow is significantly reduced (40–90% saturation) as compared with CD33 saturation in corresponding peripheral blood samples ($>$90%). *In vitro*, such reduced CD33 saturation levels were strongly related with reduced cell kill. Apparently, high CD33-antigen loads in blood consume gemtuzumab and thereby limit its penetration into bone marrow. Consequently, CD33 saturation in bone marrow is reduced, which hampers efficient cell kill. Therefore, gemtuzumab should be administered at higher or repeated doses, or, preferably, after reduction of the leukemic cell burden by classical chemotherapy.

Introduction

Gemtuzumab ozogamicin (Mylotarg®) is a humanized CD33 antibody linked to the antitumor antibiotic calicheamicin.[1] In phase-II clinical trials, gemtuzumab administered as single agent at two doses of 9 mg/m^2 2 weeks apart, resulted in complete remission in approximately 30% of relapsed AML patients.[2,3] Gemtuzumab therapy was related with low toxicity, the major complication being occurrence of hepatic veno-occlusive disease (VOD) in a small number of patients.[4] Based on these results, gemtuzumab was FDA-approved for treatment of relapsed AML patients over 60 years of age.[5]

We have previously shown that after intravenous infusion of gemtuzumab, near-complete saturation ($>$90%) of CD33-antigens is achieved on AML blast cells present in peripheral blood (PB).[6] In addition, gemtuzumab also binds to CD33-antigens expressed by monocytes and granulocytes present in PB at the time of infusion.[6] Consequently, the total amount of gemtuzumab bound to CD33-antigens in PB depends on the number of CD33-positive monocytes, granulocytes, and AML blast cells as well on the CD33 expression level of these cells (referred to as the peripheral CD33-antigen load). After binding of gemtuzumab to CD33-antigens, the CD33-antigen/gemtuzu-

mab complexes are rapidly internalized,[6] followed by hydrolysis of the acid-labile linker and subsequent activation of the calicheamicin γ_1 derivate.[1] Of importance, upon internalization of the complexes, new CD33-antigens are rapidly expressed on the cell surface and subsequently can bind gemtuzumab. The rate of renewed CD33-antigen expression is related to the degree of CD33 saturation and internalization[6] and to the activation status of the cell.[7] Consequently, the process of CD33 saturation, internalization, and new CD33-antigen expression is ongoing as long as saturating gemtuzumab concentrations are maintained. This continuous process will result in a progressive intracellular accumulation of gemtuzumab.

Obviously, high levels of intracellularly accumulated calicheamicin are more toxic than low levels. Indeed, patients with less than 5% AML blasts in their PB after the first gemtuzumab infusion had higher maximal gemtuzumab binding levels on AML blast cells (ie stronger CD33-antigen expression) than patients with more than 5% AML blasts in PB after the first infusion.[6] However, no relation was found between CD33 expression levels and clinical outcome as assessed by analysis of bone marrow (BM) samples.[2,3] Apparently, the gemtuzumab response in PB, but not in BM, is related to the CD33 expression level on AML blasts.

We hypothesized that high CD33-antigen loads in peripheral blood consume intravenously administered gemtuzumab, thereby limiting gemtuzumab concentrations in BM. As a consequence, CD33 saturation of AML blasts in BM might be reduced, resulting in greatly reduced intracellular accumulation of gemtuzumab and thereby in inefficient cell kill.

Materials and methods

Patients

Patients enrolled in the gemtuzumab phase-II clinical protocols 0903B1-202-EU ($n=54$) and 0903B1-203-EU ($n=38$) were studied.[2,3,6] In these open, single-arm multicenter phase-II studies, gemtuzumab (Wyeth-Ayerst Laboratories, St Davids, PA, USA) was administered as a single 2-h intravenous infusion at a dose of 9 mg/m^2. In general, each patient received two gemtuzumab doses with at least 14 days between the doses. Prior to the start of each gemtuzumab treatment cycle and 3 and 6 h after the start of each gemtuzumab treatment cycle, PB samples were obtained, immediately placed on ice (4°C) and shipped overnight at 4°C to Immunology, Erasmus MC, Rotterdam by express courier (World Courier, Hoofddorp, The Netherlands).

Six patients treated with gemtuzumab on compassionate use basis were also included in this study. BM samples were taken prior to and 24 h after gemtuzumab infusion. The latter sample

Table 1 Results of statistical analysis for prognostic factors

Analysis	Prognostic variable[a]	Univariate analysis[b]	Multivariate analysis[c]
CR(p) vs NR[d]	Age	0.566	n.d.
	FAB	0.508	n.d.
	Percentage blasts in BM	**0.046**	0.272
	Multidrug resistance efflux	0.888	n.d.
	ECOG performance status	0.600	n.d.
	Duration of CR1	**0.009**	0.201
	CD33-antigen load PB	**0.001**	**0.014**

[a]For details Sievers et al.[3]
[b]Mann–Whitney U-test.
[c]Logistic regression.
[d]CR(p): patients in complete remission, with or without full recovery of platelets ($n = 18$); NR: nonresponders ($n = 74$).

was immediately diluted in 200-ml ice-cold phosphate-buffered saline and kept on ice until further analysis to limit additional CD33 saturation due to the presence of gemtuzumab in contaminating PB. PB samples were taken just prior to gemtuzumab infusion and 6 and 24 h thereafter. Inclusion of additional patients treated with gemtuzumab as a single agent and at a dose of $9 \, mg/m^2$ was not possible, because current European clinical trails employ another gemtuzumab dose and/or use a combination regimen with chemotherapy.

Analysis of CD33 expression, CD33 saturation, and maximal gemtuzumab binding

Analysis of CD33 expression, CD33 saturation, and maximal gemtuzumab binding was performed as described previously using a flow cytometric assay.[6] Briefly, to determine CD33 saturation cells were incubated with biotin-conjugated mouse

anti-human IgG_4 antibodies (Caltag Laboratories, Burlingame, CA, USA), followed by streptavidin-FITC (Biosource, Nivelles, Belgium) as a second step reagent. To detect maximal gemtuzumab binding, cells were first incubated with excess gemtuzumab (final concentration: $10 \, \mu g/ml$), followed by successive incubation with biotin-conjugated mouse anti-human IgG_4 antibodies and streptavidin-FITC. As a negative control, cells were incubated with streptavidin-FITC alone. To detect CD33 antigenic sites not occupied by gemtuzumab, cells were incubated with CD33-PE (clone P67.6; BD Biosciences, San Jose, CA, USA) or, as a negative control, IgG_1-PE (BD Biosciences). All incubations were performed at 4°C. CD45-PerCP (2D1; BD Biosciences) and/or CD14-PE (My4; Coulter Clone, Hialeah, FL, USA) antibodies were included to distinguish between various cell populations.

To determine the relation between gemtuzumab concentration and CD33 saturation, the AML193 cell line or PB from healthy volunteers was incubated with various gemtuzumab concentrations (15 min at 4°C), after which CD33 saturation was analyzed.

Analysis of peripheral CD33-antigen load

Samples taken just prior to gemtuzumab infusion were subjected to routine morphological analysis to assess the leukocyte differential. In addition, maximal gemtuzumab binding to AML
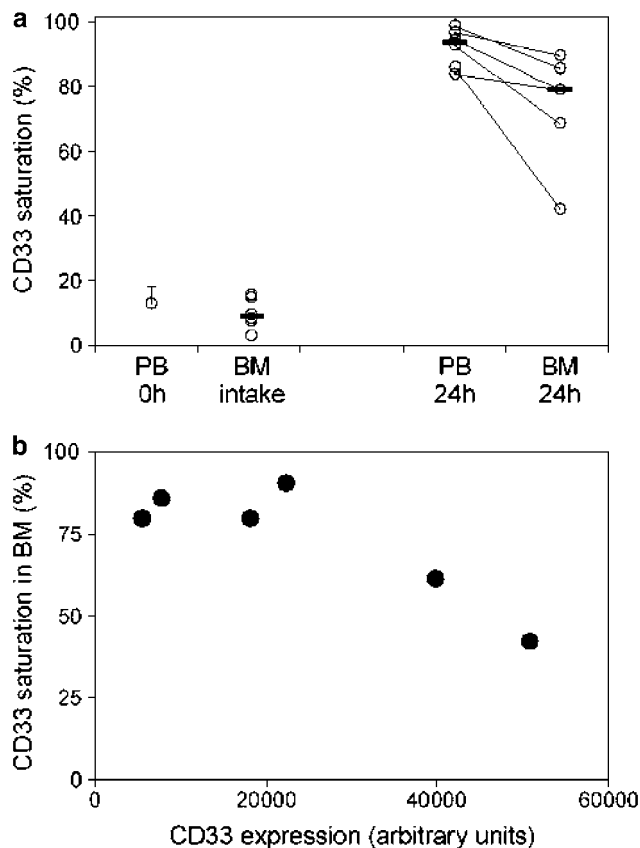

Figure 1 CD33 saturation levels in BM. (a) CD33 saturation on AML blast cells in BM and PB samples obtained prior to and 24 h after gemtuzumab infusion in six AML patients. Before gemtuzumab infusion, only background CD33 saturation levels ($<20\%$) were observed in BM. For comparison, previously published CD33 saturation levels in PB prior to gemtuzumab infusion (mean±s.d.) are shown as well.[6] At 24 h after gemtuzumab infusion, CD33 saturation levels in PB were near-complete (mean: 92%), whereas CD33 saturation levels in BM were significantly lower (mean: 74%; $P<0.05$ by Mann–Whitney test). The horizontal bars indicate the mean CD33 saturation. (b) Relation between CD33 saturation by AML blast cells in BM and CD33 expression by blast cells for the same patients. High CD33 expression levels were related to lower CD33 saturation, suggesting that also the CD33-antigen load in BM contributes to CD33 saturation in BM (Pearson's Correlation: -0.867; $P<0.05$). The six patients analyzed in our study all died: five due to disease progression and one due to stroke. Two patients (with 61 and 80% CD33 saturation in bone marrow) showed an initial response after gemtuzumab infusion, characterized by a strong reduction in blast cells in peripheral blood (to levels $<2\%$). Both patients, however, showed a fast increase in blast cells in peripheral blood 1–2 weeks later. It can be speculated that this is due to outgrowth of AML blast cells in BM, which were not sufficiently saturated with gemtuzumab for efficient induction of cell kill.

blast cells, monocytes, and granulocytes was determined as described above. The peripheral CD33-antigen load was subsequently calculated as follows: (maximal gemtuzumab binding blasts × #blasts/l) + (maximal gemtuzumab binding monocytes × #monocytes/l) + (maximal gemtuzumab binding granulocytes × #granulocytes/l).

Analysis of cell kill

To determine the relationship between induction of cell kill and gemtuzumab concentration, AML193 cells were incubated with various gemtuzumab concentrations for 24 or 40 h, after which cell kill was determined by ^{51}Cr release assay as described previously.[7,8] As AML193 cells also can take up gemtuzumab via CD33-independent mechanisms (that will contribute to its final cytotoxic activity),[7] an isotype-matched calicheamicin-conjugated antibody was not included as a control. To determine whether free calicheamicin (eg due to hydrolysis of the linkers during the incubation period) could contribute to cell kill, pilot experiments using free calicheamicin were performed. These experiments showed that the maximal amount of free calicheamicin that could be present during the incubation period ($<0.015\,\mu$g/ml at the highest gemtuzumab dose; based on less than 1% free drug in clinical grade gemtuzumab and an estimated increase with maximally 2–3% in 24 h; P Hamann, personal communication) resulted in <15% cell kill in our assay.[7]

Statistical analysis

To determine whether the peripheral CD33-antigen load was different between patients that achieved complete remission (either with or without full recovery of platelets[2,3]) and nonresponders, a Mann–Whitney U-test using log-transformed data was applied. Univariate analysis using age, FAB-type, multidrug resistance efflux, ECOG performance status, duration of CR1, and percentage BM blasts[3] was performed using the Mann–Whitney U-test. Unfortunately, karyotype data were not completely available; inclusion of fusion gene transcript data (CBFB-MYH11, PML-RARA, AML1-ETO) was not done due to the very low number of patients (three out of 92) being positive for one of these translocation-associated fusion gene transcripts. Multivariate analysis, including all indicated variables that were significant in the univariate analysis, was performed by logistic regression. In all tests, a P-value of less then 0.05 was considered significant.

Results and discussion

High peripheral CD33-antigen load is an independent prognostic factor

To evaluate whether a high peripheral CD33-antigen load was related to clinical outcome, we analyzed the CD33-antigen load in PB of 92 patients enrolled in the European phase-II protocols. The mean peripheral CD33-antigen load was significantly lower in patients who achieved complete remission than in non-responders (mean log(peripheral CD33-antigen load)±s.d.: 3.67±0.85 vs 4.58±0.97; Table 1). Further statistical analysis using age, FAB-type, multidrug resistance efflux, ECOG performance status, duration of CR1, and percentage BM blasts[3] showed that duration of CR1 and percentage BM blasts were significant prognostic markers as well (Table 1). However,
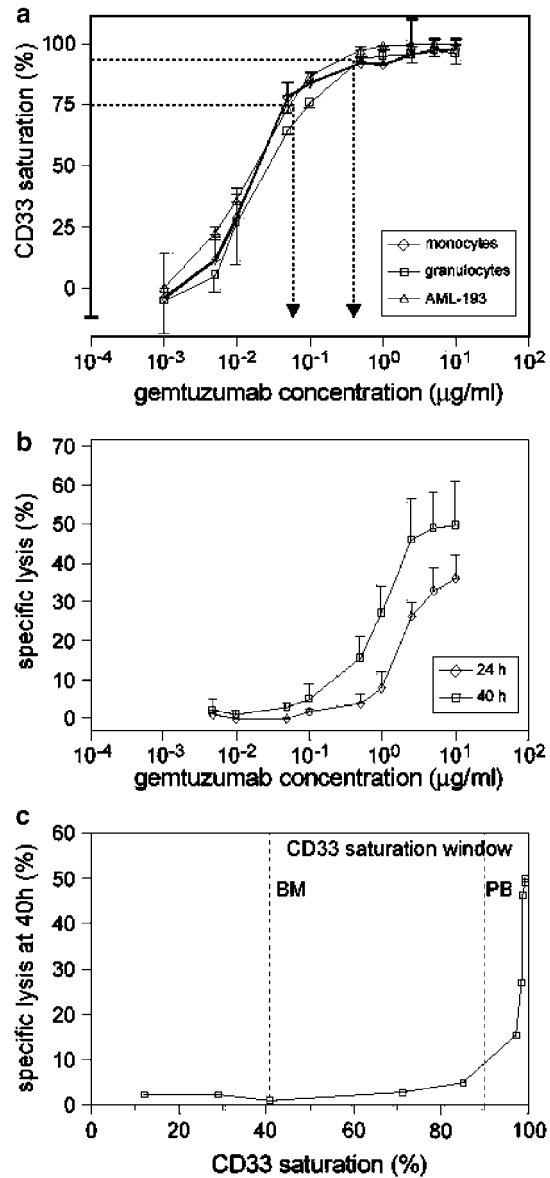

Figure 2 Relation between CD33 saturation and gemtuzumab-induced cell kill. (a) CD33 saturation of AML193 cells in relation to gemtuzumab concentration (mean±s.d.; $n=3$). For CD33 saturation levels >90% (as observed in PB), a gemtuzumab concentration of at least 0.2 μg/ml was needed. For CD33 saturation levels of 40–90% (as observed in BM), gemtuzumab concentrations of 0.01–0.1 μg/ml (ie two- 20-fold lower) were needed. The dotted lines indicate the mean CD33 saturation level in BM (74%) or PB (92%) and the corresponding gemtuzumab concentration. Comparable data were obtained for granulocytes (CD33$^{+\,(low)}$; $n=3$) and monocytes (CD33$^{+\,(high)}$; $n=3$). (b) Lysis of AML193 cells in relation to gemtuzumab concentration (mean±s.d.; $n=3$). Lysis was determined using a ^{51}Cr release assay after incubation with various gemtuzumab concentrations for 24 or 40 h.[7,8] The steep increase in cell kill observed at gemtuzumab concentration ranging from 0.1 to 2.5 μg/ml (from <5 to >45% cell kill) is assumed to be gemtuzumab-specific, because the vast majority of cell kill can be inhibited by addition of an excess of CD33-antibody.[7] At higher gemtuzumab concentrations, also CD33-independent uptake of gemtuzumab may be involved.[7] (c) Relation between CD33 saturation and cell lysis of AML193 cells. The combined data of (a) and (b) show that CD33 saturation levels >90% (as observed in PB; CD33 saturation window PB) resulted in 10–50% lysis. CD33 saturation levels <90% as observed in BM (CD33 saturation window BM) result in <5% lysis.

multivariate analysis showed that only the log(peripheral CD33-antigen load) was an independent prognostic factor (Table 1). In accordance with our data, the likelihood of achieving response to gemtuzumab therapy was shown to be associated with lower levels of CD33-positive leukemic blasts in PB[3] or BM.[2] Thus, high peripheral CD33-antigen loads reduce treatment efficacy, probably by consuming gemtuzumab and thereby reducing the gemtuzumab amount available for leukemic cells in BM. This may result in reduced CD33 saturation on AML blast cells present in BM and subsequently in inefficient induction of cell kill.
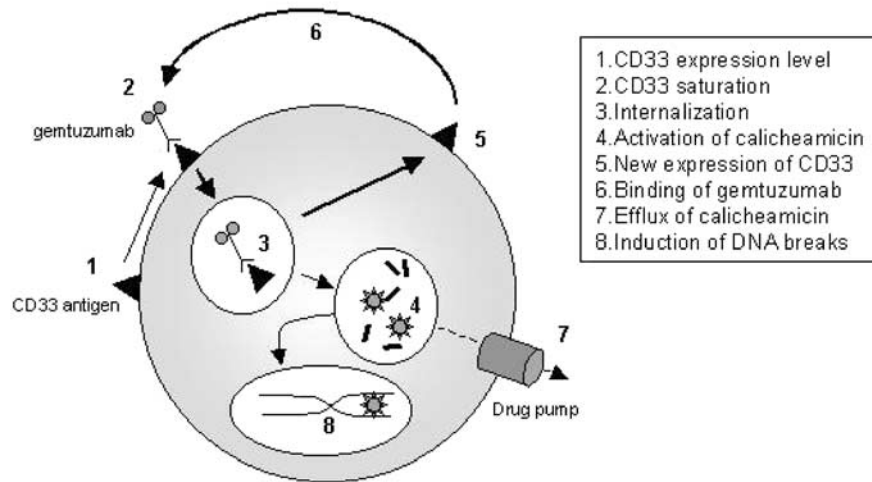
CD33 saturation on AML blast cells present in BM is reduced

To investigate whether peripheral gemtuzumab consumption indeed could reduce the gemtuzumab amount available for

leukemic cells in BM, we analyzed BM samples obtained 24 h after gemtuzumab infusion. As shown in Figure 1a, prior to gemtuzumab infusion only background saturation levels (<20%) were observed in PB and BM. In PB, near-complete CD33 saturation was observed at 6 h (data not shown) and at 24 h after gemtuzumab infusion (Figure 1a). In contrast, CD33 saturation in BM was significantly lower and ranged from 42 to 90%. Apparently, penetration of gemtuzumab (given at a dose of 9 mg/m²) into BM is not sufficient to saturate completely CD33-antigens on AML blasts present in BM 24 h after gemtuzumab infusion.

Preliminary analysis showed that the degree of reduced CD33 saturation in BM was only partly related to the peripheral CD33-antigen load (data not shown), but was related to the CD33 expression level of the AML blasts in BM (Figure 1b). It will be interesting to analyze the relationship between peripheral CD33-antigen loads and CD33 saturation in BM in future

a Cellular parameters affecting the efficacy of gemtuzumab.

1. CD33 expression level
2. CD33 saturation
3. Internalization
4. Activation of calicheamicin
5. New expression of CD33
6. Binding of gemtuzumab
7. Efflux of calicheamicin
8. Induction of DNA breaks

b Systemic parameters affecting the efficacy of gemtuzumab.

Peripheral blood Bone marrow

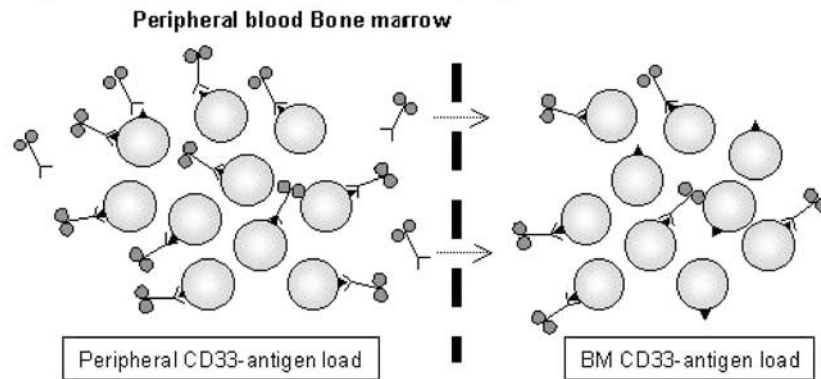Peripheral CD33-antigen load   BM CD33-antigen load

Figure 3 Parameters affecting the efficacy of gemtuzumab treatment. (a) At the cellular level, several parameters affecting gemtuzumab's efficacy can be recognized. The maximal gemtuzumab binding is dependent on the CD33 expression level of the cell (1). After gemtuzumab infusion, CD33-antigens are rapidly saturated (2) and the gemtuzumab/CD33-antigen complexes are subsequently internalized (3). In lysosomes, the acid-labile linker is hydrolyzed, followed by activation of calicheamicin and degradation of the antibody part of gemtuzumab (4). Upon internalization, new CD33-antigens are rapidly expressed on the cell membrane (5) and can bind gemtuzumab (6), resulting in an ongoing process of gemtuzumab internalization as long as saturating gemtuzumab concentrations are present. Calicheamicin can be pumped out of the cell by drug pumps such as Pgp and MRP1 (7). Active calicheamicin enters the nucleus and can induce double-strand DNA breaks (8), finally resulting in the induction of cell death. (b) Besides cellular parameters, systemic parameters are involved in the efficacy of gemtuzumab. These particularly include the peripheral CD33-antigen load, which determines the amount of gemtuzumab that is consumed by the myeloid cells in PB and consequently the amount of gemtuzumab available for binding to CD33-antigens in BM. CD33 saturation in BM also appeared to be affected by the total CD33-antigen load in BM.

protocols involving gemtuzumab in more detail. However, it should be kept in mind that the final CD33 saturation level in BM is not only affected by the peripheral CD33-antigen load, but may also be dependent on the total CD33-antigen load in BM.

Complete saturation is required for efficient cell kill

After binding to CD33-antigens, gemtuzumab is rapidly internalized and the calicheamicin derivate is intracellularly activated.[1,6] Importantly, continuous renewed expression of CD33-antigens on the cellular surface of myeloid cells significantly increases the amount of internalized gemtuzumab as long as saturating levels of gemtuzumab are present.[6] Therefore, the reduced CD33 saturation levels observed in BM may result in severely reduced accumulation of gemtuzumab into the cell, and thereby in inefficient induction of cell kill. To evaluate whether reduced CD33 saturation levels indeed resulted in less efficient induction of cell kill, myeloid cells (monocytes, granulocytes, AML193 cells) were incubated with gemtuzumab, and CD33 saturation and/or cell kill were determined. As shown in Figure 2, CD33 saturation and gemtuzumab-induced cell kill were both concentration dependent. Of importance, the combined CD33 saturation and cell kill data indicate that CD33 saturation levels $<90\%$ (as found in BM) resulted in a dramatic decrease in cell lysis ($<5\%$; Figure 2c). Thus, gemtuzumab concentrations resulting in near-complete CD33 saturation levels ($>90\%$) are required for efficient cell kill. In our opinion, this reflects the continuous process of saturation, internalization, and new expression of CD33-antigens,[6,9] resulting in the progressive accumulation of gemtuzumab (and consequently calicheamicin) in the cell (see Figure 3a). In agreement with this, the steep increase in gemtuzumab-induced cell kill (from 15 to $>45\%$) is observed in the gemtuzumab concentration range of 0.5–$2.5\,\mu g/ml$; at these concentrations the vast majority of the gemtuzumab-induced cell kill is CD33-mediated.[7] Additionally, at such high gemtuzumab concentrations ($>1\,\mu g/ml$) CD33-independent uptake mechanisms (ie endocytosis) may occur.[7] Irrespective whether gemtuzumab is taken up by the cell via CD33-dependent internalization or via CD33-independent mechanisms, our data indicate that high gemtuzumab concentrations (those resulting in near-complete CD33 saturation) are needed for efficient cell kill. The reduced CD33 saturation levels in BM (Figure 1) indicate that the gemtuzumab concentration in BM is relatively low, and consequently cell kill will be reduced (Figure 2).

Implications for gemtuzumab therapy

In this report, we show that a high peripheral CD33-antigen load is an independent adverse prognostic factor in AML patients treated with gemtuzumab. Our data indicate that a high peripheral CD33-antigen load consumes a large part of the gemtuzumab dose ($9\,mg/m^2$), which results in reduced gemtuzumab penetration in BM (Figure 3b). As a consequence, CD33 saturation of AML blast cells present in BM is incomplete, which drastically hampers subsequent cell kill.

For efficient killing of AML blasts in BM, the effective gemtuzumab dose should be increased in order to reach consistently high CD33 saturation levels. This can be done by increasing the dose of $9\,mg/m^2$ or by giving repeated doses at subsequent days,[9] which may however be complicated by the increase of severe side effects, including VOD.[4] Alternatively and preferably, gemtuzumab could be administered *after* reduction of the leukemic cell burden by classical chemotherapy. However, so far, in most studies applying gemtuzumab-based combination regimens, gemtuzumab is given prior to or simultaneously with chemotherapy.[10–15] The use of gemtuzumab in a postremission regimen, combined with chemotherapy, has recently shown to be feasible and well-tolerated but needs further investigation.[16] To further improve the intracellular accumulation of gemtuzumab, drug efflux modulators such as cyclosporine A may be added to the treatment regimen as well.[13–18] A better understanding of gemtuzumab's mechanism of action will hopefully contribute to the design of future treatment protocols that maximally exploit the potencies of gemtuzumab.

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