Gemtuzumab Ozogamicin (GO) Inclusion to Induction Chemotherapy Eliminates Leukemic Initiating Cells and Significantly Improves Survival in Mouse Models of Acute Myeloid Leukemia

Cathy C. Zhang*, Zhengming Yan*, Bernadette Pascual*, Amy Jackson-Fisher*, Donghui Stephen Huang†, Qing Zong†, Mark Elliott‡, Conglin Fan*, Nanni Huser*, Joseph Lee*, Matthew Sung‡ and Puja Sapra‡

*Pfizer Worldwide Research and Development, Oncology Research Unit, La Jolla, CA; †Pfizer Worldwide Research and Development, Drug Safety Research and Development Group, La Jolla, CA; ‡Oncology Research Unit, Pearl River, NY

Abstract

Gemtuzumab ozogamicin (GO) is an anti-CD33 antibody-drug conjugate for the treatment of acute myeloid leukemia (AML). Although GO shows a narrow therapeutic window in early clinical studies, recent reports detailing a modified dosing regimen of GO can be safely combined with induction chemotherapy, and the combination provides significant survival benefits in AML patients. Here we tested whether the survival benefits seen with the combination arise from the enhanced reduction of chemoresidual disease and leukemic initiating cells (LICs). Herein, we use cell line and patient-derived xenograft (PDX) AML models to evaluate the combination of GO with daunorubicin and cytarabine (DA) induction chemotherapy on AML blast growth and animal survival. DA chemotherapy and GO as separate treatments reduced AML burden but left significant chemoresidual disease in multiple AML models. The combination of GO and DA chemotherapy eliminated nearly all AML burden and extended overall survival. In two small subsets of AML models, chemoresidual disease following DA chemotherapy displayed hallmark markers of leukemic LICs (CLL1 and CD34). In vivo, the two chemoresistant subpopulations (CLL1+/CD117− and CD34+/CD38+) showed higher ability to self-renewal than their counterpart subpopulations, respectively. CD33 was coexpressed in these functional LIC subpopulations. We demonstrate that the GO and DA induction chemotherapy combination more effectively eliminates LICs in AML PDX models than either single agent alone. These data suggest that the survival benefit seen by the combination of GO and induction chemotherapy, nonclinically and clinically, may be attributed to the enhanced reduction of LICs.

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Introduction

Acute myeloid leukemia (AML) is the most common acute leukemia in adults. Treatment options for AML are limited, with standard first-line therapy consisting of a chemotherapy combination including cytarabine and an anthracycline (daunorubicin most commonly used). Despite favorable complete remission rates in young and old patients, early relapsed disease is common, and the prognosis of relapsed AML patients is poor as 5-year survival rates are low (30% in patients <60 years, 5%–10% in patients ≥60 years).

Gemtuzumab ozogamicin (GO) is a CD33-antibody-drug conjugate (ADC) composed of a humanized monoclonal antibody linked to the potent cytotoxic agent calicheamicin via a hydrazone linker. GO binds

Abbreviations: GO, gemtuzumab ozogamicin; AML, acute myeloid leukemia; DA, daunorubicin/cytarabine; NSG, nonobese diabetic severe combined immunodeficiency gamma; PDX, patient-derived xenograft; LIC, leukemic-initiating cell; PB, peripheral blood; BM, bone marrow; IV, intravenous

Address correspondence to: Cathy C. Zhang, Oncology Research Unit, Pfizer Global R&D, 10724 Science Center Dr., San Diego, CA 92121.

E-mail: cathy.zhang@pfizer.com

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the AML antigen CD33 and, upon internalization, releases its DNA-damaging warhead to kill the target AML cells [1]. CD33 is ubiquitously expressed on AML myeloblasts including those responsible for relapsed disease [2]. Mylotarg received FDA approval in 2000 for CD33-positive AML patients but was voluntarily withdrawn in 2010 due to safety concerns. However, four other large trials were pursued simultaneously evaluating the combination of lower-dose GO with induction chemotherapy [3]. In a collective meta-analysis of individual patient data from these trials, the addition of GO to induction chemotherapy did not affect complete remission rates; however, it significantly reduced the risk of relapse and improved the relapse-free survival and overall survival of adult AML patients with favorable cytogenetics [3]. These data suggest that the inclusion of GO to induction chemotherapy results in more durable remissions in AML patients treated with the combination.

The poor long-term prognosis of standard induction (DA) therapy correlated with the levels of minimal residual disease (MRD) [4]. A high level of MRD was found to correlate with a higher percentage of chemoresistant leukemic stem cells, which are characterized by their inherent ability for self-renewal, in the bone marrow (BM) of AML patients treated with induction therapy [5,6]. The resistance to standard care chemotherapy and the ability of leukemic-initiating cells (LICs) to initiate AML have been extensively studied. LICs were found to be a significant predictor of clinical outcome and patient survival in AML [7,8].

Herein, we hypothesize that the combination of low-dose GO and induction chemotherapy achieves more durable remissions by more effectively clearing minimal residual disease, including LICs, than either component separately. We utilize in vivo cell-line xenograft and patient-derived xenograft (PDX) AML models to demonstrate the impact on overall efficacy and duration of response that the combination of low-dose GO and induction chemotherapy provides. In so doing, we provide evidence that this combination can target cells responsible for chemorefractory disease.

Materials and Methods

GO (Mylotarg, Pfizer, New York, NY) was diluted in deionized water, and cytarabine (Pfizer, New York, NY) and daunorubicin (Teva Parenteral Medicines) were diluted with phosphate-buffered saline and stored at 4°C before usage. 8.8 ADC is a nonbinding antibody conjugated to N-Ac-γ-calicheamicin DMH (Pfizer, New York, NY). AC220 was purchased from Selleck Chemicals (Houston, TX) and prepared as previously described [9].

Flow Cytometry

Cell surface marker staining procedures were performed according to the antibody manufacturer’s instructions. Freshly prepared cells were analyzed on a BD FACSCalibur Flow Cytometer equipped with BD CellQuest Pro (BD Bioscience, San Jose, CA). Quadrant markers were set relative to negative immunoglobulin isotype controls. The percent of human AML engraftment was defined as the percentage of the human CD33+/CD45+ cells relative to the total number of BM cells. AML engraftment was also monitored by quantifying the population of human CD33+/CD45+ cells in peripheral blood (PB). Individual subpopulations were gated on live human CD45+ cells. The antibodies used were anti–hCD33-APC and anti–hCD117-APC (Thermo Fisher Scientific, San Diego, CA), anti–hCD45-FITC and anti–hCD38–FITC (BD Biosciences, San Jose, CA), anti–hCD3–PE (Miltenyi Biotec Inc., San Diego, CA), and anti–hCLL1-PE (Biolegend, San Diego, CA).

AML Models

Female NSG (NOD/SCID IL2Rγ−/−, Jackson Laboratories, Bar Harbor, ME) mice (7-8 weeks old) were used in all studies in this report. All experimental animal procedures complied with the Guide for the Care and Use of Laboratory Animals (Institute for Laboratory Animal Research, 2011) and were approved by the Institutional Animal Care and Use Committee (IACUC).

MV4-11 cells were purchased from ATCC (Manassas, VA) and stably transfect with luciferase (Luc) before implantation to generate the MV4-11-Luc cell line. For the MV4-11-Luc model, 1 × 10⁶ cells were implanted intravenously (IV) into the tail vein of nonirradiated mice. Leukemic disease progression was monitored as previously described [10] on an IVIS200 system (PerkinElmer, Waltham, MA). Treatment was initiated when the mean value of bioluminescence intensity (BLI) in each group reached approximately 5 × 10⁷ photons/sec.

To establish the BM0407 (BM120407L) and BM2407 (BM012407L) PDX models, the xenografted AML mice with P1 passage were purchased from Jackson Laboratory (Farmington, CT) and served as donors for PDX expansion. BM0407 and BM2407 were derived from FLT3-ITD–positive patients with the FAB-M2 subtype. BM0407 also harbors NPM1 mutation (exon 12 insertion). Once engrafted in mice, BM AML blasts were deep sequenced with a cancer gene panel using multiplex amplicon technologies [11] to confirm the molecular profiles. One to 2 × 10⁶ viable AML cells were collected from the BM of donor mice and were injected via the tail vein after the NSG mice were sublethally irradiated (150 cGy) using the X-Rad 225Cx (Precision X-Ray Inc., North Branford, CT). Engraftment was tracked periodically by quantifying the population of human CD45+ AML cells in the PB with flow cytometry. When the mean engraftment levels reached 1% to 5% CD33+/CD45+ cells in the PB, leukemic mice were randomly assigned to receive treatments.

In Vivo Treatment and Antileukemic Efficacy Assessment

The maximum tolerated dose of GO in AML mice is 0.3 mg/kg (IV). The plasma level in this dose is within the range of the clinical exposure of high-GO dosing regimen (9 mg/m²). To mimic the clinical strategy of fractionated GO regimen, we used low dose levels of GO (0.01 mg or 0.06 mg) for combination studies. The exact dose level for each model was preoptimized to avoid the nonspecific activity by control ADC. For each treatment cycle of the daunorubicin and cytarabine (DA) doublet arm, mice received daunorubicin intravenously on days 1, 3, and 5 and cytarabine subcutaneously on days 1 to 5. MV4-11-Luc and other models received one DA treatment cycle. BM2407 and BM0407 received two cycles of DA treatment in the combination test. AC220 was orally administered daily for 12 days at 10 mg/kg, which is a biologically effective dose for inhibiting the target (phospho-FLT3) [9]. The antileukemic efficacy was evaluated in the BM for the presence of human CD33+/CD45+ cells 5 days after the last treatment.

Assessment of Self-Renewal Ability of the Residual Cells After Induction Therapy

Bone marrow cells were collected from engrafted BM2407–diseased mice, sorted into CD34+ and CD34– subpopulations, and injected intravenously into sublethally irradiated recipient mice (n = 5). Similar methods were used for the self-renewal analysis.
of CLL1+CD117+ and CLL1+CD117− subpopulations from the BM0407 model. The mice were monitored for disease development in PB followed by BM collection.

### Data Analysis
Statistical analyses were conducted using GraphPad Prism (La Jolla, CA) with one-way analysis of variance followed by a log-rank (Mantel-Cox) test.

**Figure 1.** Antileukemic efficacy of DA chemotherapy and a selective FLT3 inhibitor AC220 in FLT3-ITDmut AML xenografts. Leukemic mice were randomly assigned to three groups and treated with 1) vehicle; 2) AC220, 10 mg/kg, po daily for 12 days; or 3) DA doublet therapy at the MTD (daunorubicin, 1.5 mg/kg, IV on days 1, 3, and 5; cytarabine, 15 mg/kg, SC on day 1-5). On day 26 to 28 after the first dosing, flow cytometry was performed to assess the AML engraftment or percentage of hCD33+/CD45+ cells in the PB and BM. n = 5 per group. To set gates for flow cytometry analysis, more than 98% of the cells stained with isotype control antibody fell within the bottom left quadrant. (A) Representative images depict the engraftment of human CD33+/CD45+ leukemic cells in the PB and BM in the different AML models following drug treatment. (B) Quantitative measurement of the mean BM AML disease burden (hCD33+/CD45+ cells relative to the total cells) posttreatment. The bar graph shows the mean relative values of the controls (100%). Values = mean ± S.E.M. *P < .05.
Results

Characterization of In Vivo AML Models and Their Response to Induction Chemotherapy and FLT3-Targeted Agent AC220

We evaluated the antileukemic efficacy of daunorubicin and cytarabine chemotherapy (DA) and a selective FLT3 inhibitor, AC220 [12,13], in three disseminated AML models including MV4-11-luc, BM2407, and BM0407 that harbor FLT3 mutations (Figure 1, A and B). The DA doublet dosing regimen was the maximum tolerated dose (MTD). AML burden was evaluated as the percentage of CD45+/CD33+ cells in the BM compartments following drug treatment. In MV4-11-Luc leukemic mice, DA treatment significantly (P < .05) reduced the percentage of AML blasts in BM compared to vehicle-treated mice (27% CD45+/CD33+ DA vs 76% CD45+/CD33+ vehicle). AC220 treatment completely eliminated MV4-11-Luc blasts in BM (0% CD45+/CD33+). We also similarly evaluated two AML PDX models (BM2407 and BM0407) from patients with FLT3-ITD–positive disease. DA treatment reduced BM CD45+/CD33+ blasts in both PDX models, albeit at different magnitudes (56% DA vs 99% vehicle in BM2407 and 11% DA vs 89% vehicle in BM0407). AC220 treatment also reduced BM CD45+/CD33+ blasts in the PDX models but not to the degree demonstrated in the MV4-11-Luc model (52% AC220 vs 99% in BM2407 and 52% AC220 vs 89% in BM0407). In the BM2407 model, we monitored AML blasts in PB and observed near-complete elimination of AML blasts in PB in both DA and AC220 treatment regimens.

In all three models that harbor the FLT3-ITD mutation, only MV4-11-Luc showed complete BM depletion after AC220 monotherapy. DA chemotherapy at the MTD showed only partial reduction in the BM blast counts of all tested AML xenografts. Of note, the plasma exposures of DA doublet were within the range of clinical exposures.

Assessing the Combinatorial Benefits of GO and DA Induction Chemotherapy in MV4-11-Luc AML Mice

Since the chemorefractory disease following DA doublet therapy was CD33+, we hypothesized that the addition of the anti-CD33 ADC (GO) to DA doublet therapy would improve the antileukemic response in the MV4-11-Luc model. We longitudinally monitored MV4-11-Luc leukemic mice with bioluminescence imaging following treatment with the DA and GO therapies as single agents and in combination (Figure 2A). On day 25 following the initial dose of the compounds, DA (32%) and GO (22%) alone reduced leukemic burden via bioluminescence imaging (Figure 2B) compared to vehicle (100%) or control ADC (100%) treatments. Importantly, only the DA and GO combination group had no detectable disease (0%). Similarly, DA (29%) and GO (32%) alone reduced the amount of CD45+/CD33+ blasts in the BM of these mice compared to vehicle (76%) or control ADC (77%) treatments (day 26), but only the DA and GO combination group (0.13%) resulted in the near-complete elimination of BM CD45+/CD33+ blasts (Figure 2, B and C).

To evaluate the duration of this enhanced response, we performed a similar study with the same dosing paradigm utilizing hind-limb paralysis as a survival readout (Figure 2, D). In this model, vehicle-treated mice have a median survival of 23 days. DA and GO monotherapy groups increased the median survival to 34 and 31 days, respectively. Mice from these groups eventually developed hind-limb paralysis. However, the DA and GO combination group revealed an extraordinary survival benefit as the median survival was not reached with four out of the five animals alive until the end of the study (day 73). By testing treatment effects on BM AML clearance and hind-limb paralysis survival, we demonstrate the remarkable combination benefit of GO and DA compared to either single agent in the MV4-11-luc model.

Antileukemic Efficacy and Survival Benefits of GO and DA Combination Observed in AML PDX Models

We employed similar study designs in the two AML PDX models to determine if the benefits observed with addition of GO to induction chemotherapy could be extended into the more chemorefractory PDX models. Mice bearing BM0407 AML were dosed with the monotherapy and combination regimen for one or two treatment cycle (Figure 3, A). On day 21 (after one cycle of treatment), CD45+/CD33+ blasts were measured via flow cytometry from BM harvests. Control ADC showed no effect with 83% CD45+/CD33+ blasts in the BM compared to vehicle (82%). GO and DA monotherapy (concurrent dosing) partially inhibited leukemic growth with 34% and 53% CD45+/CD33+ blasts remaining, respectively (Figure 3, C). Intriguingly, the combination of GO and DA resulted in near-complete elimination of CD45+/CD33+ blasts in the BM with 0.1% blasts remaining. In a second cohort of animals that received two cycles of treatment, GO treatment resulted in a similar reduction of CD45+/CD33+ blasts as the first cycle (30%), whereas the DA treatment further reduced the CD45+/CD33+ (16%) blasts compared to the first cycle (Figure 3, B and C). Strikingly, the combination of GO and DA completely eliminated all CD45+/CD33+ AML PDX blasts from the BM.

To further determine the enhanced response with the combination, we assessed if concurrent or sequential addition of GO to the DA therapy impacted the survival. In the BM0407 PDX model, the median survival in vehicle-treated mice was 48 days (Figure 3D). GO and DA monotherapy regimens prolonged survival of mice with median survival time as 67 and 92 days, respectively; however, mice in these groups eventually succumbed to disease burden or hind-limb paralysis. Impressively, the addition of GO to DA therapy, both concurrently and sequentially, completely prevented any mice from developing AML-driven hind-limb paralysis for the duration of the study (~6 months). These data suggest that the addition of GO to DA, either concurrently or sequentially, enhances the antileukemic activity of either monotherapy in AML with poor prognostic genes.

Similar results were observed in the BM2407 PDX model (Figure 3, E and F). We observed complete elimination of BM CD45+/CD33+ blasts, whereas the DA or GO treatment only showed partial inhibition against leukemic growth.

Molecular Characterization of AML PDX Models Highlights the Phenotypic Diversity of the Disease

The genetic heterogeneity of AML had been well established since the development of karyotyping techniques in the late 1970s [14,15]. As mentioned earlier, BM0407 and BM2407 both were confirmed for FLT3-ITD positivity. Additionally, BM0407 harbors AML-relevant mutations in the DNMT3A and NPM1 genes which were conserved compared to the primary patient samples.

In AML, disease remission and relapse following induction chemotherapy are thought to be mediated via the presence of chemorefractory LICs. There are several putative cell surface markers
of AML LICs including CD33, CD123, CD34, CLL1, and CD117. We performed immunophenotyping of the three AML models used in this report to screen for differences in the expression profiles of these cell surface markers. The MV4-11-Luc model was positive for CD33 and CD123 surface expression with moderate expression of CLL1 and CD117 (Figure 4). The PDX BM0407 and BM2407 models were CD33+, CD123+, CD117+, and CLL1+ (Figure 4). The PDX BM2407 model showed a CD34+ subpopulation (Figure 4).

Despite the genetic and phenotypic heterogeneity, all tested AML models express high levels of CD33 on the surface. CD33 expression may be the primary determinant for receiving the additional benefit of GO with induction chemotherapy in these AML models.

**DA Induction Therapy Enriches for a CLL1+/CD117− Subpopulation in Residual Disease**

Following the molecular characterization of our AML models, we hypothesized that the residual disease following DA induction therapy enriched the AML models for CD45/CD33+ cells expressing putative markers of LICs. In BM0407, DA chemotherapy reduced the CD45/CD33+ blast population in BM to 20% as compared with 80.1% in vehicle-treated mice (Figure 5A, top). However, the
Figure 3. GO in combination with DA chemotherapy exhibits a synergistic antileukemic effect and improves survival in BM0407 AML PDX models. BM0407 leukemic mice were randomly assigned before treatment when the mean engraftment levels reached 1% to 5% CD33+/CD45+ cells in the PB. Mice were treated with 1) vehicle; 2) negative control ADC; 3) GO; 4) DA chemotherapy (cytarabine at 10 mg/kg SC on days 1-5 and 22-26 and daunorubicin at 1 mg/kg IV on days 1, 3, 5, 22, 24, and 26); 5) GO and DA concurrently with DA therapy; or 6) GO following DA therapy. GO or negative control ADC was injected IV at 0.06 mg/kg on days 1 and 22. On days 21 and 30 after the initial dosing, two sets of BM0407 PDX mice were euthanized to evaluate the BM disease burdens by flow cytometry analysis of CD33+/CD45+ cells (B, C). (A) Schematic of study design and dosing regimens. (B) Representative evaluation of CD33+/CD45+ disease burden in BM following drug treatment on day 21. (C) Quantification of AML blast burden across all treatment groups. Values = mean ± S.E.M. *P < .05. (D) In a separate cohort of animals, mice were monitored for development of hind-limb paralysis as a surrogate for survival with AML disease. Mice were monitored for 180 study days. n = 5 mice per group. Concurrent or sequential treatment of DA and GO significantly (P < .05) improved survival by either GO or DA monotherapy. (E) GO in combination with DA chemotherapy exhibits a synergistic antileukemic effect and improves survival in BM2407 AML PDX model. BM2407 leukemic mice were randomly assigned before treatment when the mean engraftment levels reached 1% to 5% CD33+/CD45+ cells in the PB. Mice were treated with 1) vehicle; 2) negative control ADC; 3) GO; 4) DA chemotherapy (cytarabine at 15 mg/kg SC on days 1-5 and days 19-23; daunorubicin at 1.5 mg/kg IV on days 1, 3, 5 and days 19, 21, 23); or 5) GO or negative control ADC injected IV at 0.06 mg/kg on days 1 and 19. On day 28 after the initial dosing, mice were euthanized to evaluate the BM disease burdens by flow cytometry analysis of CD33+/CD45+ cells (A, B). (A) Representative evaluation of CD33+/CD45+ disease burden in BM following drug treatment on day 28. (B) Quantification of AML blast burden across all treatment groups. Values = mean ± S.E.M. n = 5 mice per group. *P < .05.
CD45+/CD33+ blasts were significantly \( (P < .05) \) enriched by 137% for a CLL1+/CD117− subpopulation following DA induction chemotherapy (49.7%) as compared to vehicle-treated mice (37.6%) (Figure 5A, bottom). Other AML models demonstrated even greater enrichment for CLL1+/CD117− blasts following DA induction chemotherapy (222% in AML124 and 157% in MV4-11-luc) (Figure 5B).

To assess the self-renewal capability of the different populations of CD45+/CD33+ AML blasts, we sorted purified CLL1+/CD117− and CLL+/CD117+ AML blasts from mice engrafted with BM0407 and reimplanted these two subpopulations of cells into naïve recipient mice and monitored for BM engraftment of AML cells (Figure 5C). We implanted 10,000 (1e4) or 100,000 (1e5) of each cell subpopulation into five mice and collected BM from the implanted mice after 113 days to measure the amount of CD45+/CD33+ blast engraftment in the BM (Figure 5D). All mice (5/5) implanted with 1e5 CLL1+/CD117− cells had BM leukemic engraftment, but very low engraftment was observed in mice implanted with 1e5 CLL1+/CD117+ cells (Figures 5D and 1E). Mice implanted with 1e4 CLL1+/CD117− or CLL1+/CD117+ cell displayed similar trend of BM engraftment (0.1% vs 0%). These observations suggest that CLL1+/CD117− subpopulation is more chemorefractory and leukemogenic in the BM0407 AML PDX model.

**DA Induction Therapy Enriches for a CD34+/CD38+ LIC Subpopulation in Residual Disease**

In a similar set of experiments as described earlier, we assessed the capability of DA induction chemotherapy to enrich for a CD34+ subpopulation in residual disease in AML models. In BM2407, DA induction chemotherapy significantly \( (P < .05) \) reduced the CD45+/CD33+ blast population in BM to 7% as compared with 97% in vehicle-treated mice (Figure 6A, top). The CD45+/CD33+ blasts were enriched by 164% for a CD34+ subpopulation following DA chemotherapy (56%) as compared to vehicle-treated mice (33%) (Figure 6, A and B). Other AML models demonstrated similar or greater enrichment for CD34+ blasts following DA induction chemotherapy (Figure 6B).

To assess the self-renewal capability of these two different CD33+ AML subpopulations, we sorted CD34+ and CD34− AML blasts from mice engrafted with BM2407 and reimplanted these two subpopulations of cells into mice and monitored for BM engraftment of AML cells (Figure 6C). We implanted 10,000 (1e4), 200,000 (2e5), or 600,000 (6e5) of each cell subpopulation into five mice and collected BM from the implanted mice after 172 days for the mice implanted with 1e4 cells and 97 days for other transplanted mice (2e5 and 6e5) to measure the amount of CD45/CD33+ blast engraftment in the BM (Figure 6D). In this setting, all mice (5/5) implanted with 6e5 or 2e5 and two of five mice implanted with 1e4 CD34+ cells had BM leukemic engraftment, but no engraftment (0/5) was observed with implantation of 1e4, 2e5, or 6e5 CD34− AML blasts (Figure 6E). In this model, both CD34+ and CD34− cells express CD38. Thus, CD34+/CD38+ cells appear to be a marker of LICs from the BM2407 AML PDX model, and the CD34+/CD38+ LICs are enriched in residual disease following DA induction therapy.

**Discussion**

Although most AML patients achieve complete remission after induction therapy, the overall survival rate remains poor [16]. In this report, the combination of GO and DA chemotherapy exhibited synergistic effects in AML xenografts. Importantly, GO was tested at levels that were five-fold lower than the MTD for chemocombination to determine a correlation with the clinical dosing strategy. Over the past decade, GO has been shown to be most effective in AML patients at lower doses with more frequent administration [17]. In addition, a PK/PD-guided dosing schedule that is optimized in patients [18] further supports a new dosing regimen strategy based on saturable
receptor occupancy at a lower dose of GO [19]. With the newly developed dosing regimen, GO demonstrated benefits in AML patients either as monotherapy or in combination with other agents [20]. Our data further support this clinical strategy. Low-dose GO treatment combined with DA therapy, via either concurrent or sequential administration, significantly improved the survival rate compared to DA chemotherapy at the MTD. In addition, AML patients with FLT3-ITD have been reported to have poor cure rates [21]. Although FLT3 inhibitors have had some positive effect, drug resistance and dose limiting toxicity have caused challenges for clinical development [22]. Here, combined GO and DA treatment provided significant benefits over the FLT3 inhibitor AC220 in all three xenograft models with FLT3-ITD mutations, suggesting a possible broader application in AML patients.

LICs are believed to be one of the leading causes for AML treatment failure and relapse after chemotherapy [23]. Abundant research has suggested that LICs express heterogeneous surface markers. Previous reports have shown that AML LICs primarily exist as a CD34+/CD38− subpopulation. In recent years, studies have demonstrated that LICs also reside in the CD34+/CD38+ or even in

Figure 5. DA chemotherapy enriches a CLL1+/CD117− subpopulation in BM0407 residual disease. (A) BM0407 leukemic mice were treated with vehicle or DA doublet therapy (daunorubicin at 1.5 mg/kg IV on days 1, 3, and 5; cytarabine at 15 mg/kg SC on days 1-5). On day 28, flow cytometry analysis was performed to measure the CD33+/CD45+ cells in the BM of the leukemic mice (top). The fraction of CLL1+/CD117− cells was enriched in the AML-gated cells (bottom). (B) Two additional models, AML124 and MV4-11-Luc, were also tested for the effect of DA treatment on the CLL1+/CD117− subpopulation. Values for all three models were normalized to the corresponding control. n = 5 mice per group. *P < .05. (C) Study design for the isolation and reimplantation of CLL1+/CD117− and CLL1+/CD117+ cells using freshly collected BM cells from the BM0407 model. D) Representative image of CD33+/CD45+ blasts from the BM of mice engrafted with CLL1+/CD117− or CLL1+/CD117+ cells. (E) BM collection and analysis were performed at 113 days after AML transplantation into immunocompromised mice injected with 1 × 10^4 (1e4) or 1 × 10^5 (1e5) sorted CLL1+/CD117− or CLL1+/CD117+ cells. Values = mean ± S.E.M. n = 5 mice per group.
the CD34− state [8,24,25]. Consistent with these findings, we observed the self-renewal potential of the CD34+ subpopulation in the PDX model BM2407 that fully expresses CD38; i.e., CD34+/CD38+ was able to initiate AML in NSG mice. In another PDX model, BM0407, which lacks CD34 expression and highly expresses CD38 (98%) of the AML cells, we demonstrated the AML repopulating ability of the CLL1+/CD117− subpopulation in NSG mice. Interestingly, PDX BM0407 lost CD34 expression after xenotransplantation, although it was partially expressed in the patient sample, which suggests that the LIC subpopulation may vary among individuals [26]. We also noticed that the CD34+ LIC subpopulation from BM2407 PDX fully expresses CLL1. Multiple studies have

**Figure 6.** DA chemotherapy enriches a CD34+ subpopulation in BM2407 residual disease. (A) Representative images from flow cytometry analysis of BM2407 cells from BM post-DA treatment. BM2407 leukemic mice were treated with vehicle or DA doublet therapy (daunorubicin at 1.5 mg/kg IV on days 1, 3, and 5; cytarabine at 15 mg/kg SC on days 1-5). On day 28 after the initial dose, BM cells were collected from the leukemic mice for flow cytometry analysis. DA treatment significantly reduced leukemic burden in the BM (top), and CD34+ cells were enriched in the AML-gated cells (bottom). (B) Two additional models, AML146 and the AML 124, also showed enrichment of CD34+ cells in BM residual disease. Values for all three models were normalized to the corresponding control. *P < .05. (C) Study design for the isolation and reimplantation of CD34+ and CD34− cells using freshly collected BM cells from the BM2407 model. Cells were stained and sorted into CD34+ and CD34− subpopulations prior to being reimplanted into the irradiated recipient mice. (D) Representative image of CD33+/CD45+ blasts from the BM of mice engrafted with CD34+ or CD34− cells. (E) BM collection and analysis were performed at day 172 for the mice that received the 1 × 10⁴ transplanted cells and at day 97 for remaining transplanted mice. Bar graphs show the leukemic repopulation of CD34− or CD34+ subpopulations when different doses of cell were reimplanted. Values = mean ± S.E.M. n = 5 mice per group.
demonstrated that, in AML patient specimens, CLL1+ cells are present in the LIC fraction [27,28] but are absent from normal or regenerating BM cells. In addition, CLL1+ cells were associated with rapid disease relapse [28]. However, how CLL1+ cells function as LICs has not been reported. To our knowledge, this is the first study to show the self-renewal ability of CLL1+/CD117− cells isolated from AML PDX models.

Chemoresistance has been the leading cause of AML treatment failure despite the high initial response rate. In the subset of AML models we tested, human AML cells in the PB showed significantly higher sensitivity to DA chemotherapy than did those in the BM. However, despite a modest decrease in BM disease burden, DA-treated AML mice showed no overall survival benefit because the disease eventually relapsed in all three models. Clearly, the residual AML cells in the BM became cell cycle quiescent and failed to respond to chemotherapy due to the protection provided by the BM niche [7,29]. We hypothesize that recurrence of the disease may arise from self-renewal of LICs present in the chemoresistant residual disease. This notion is supported by the assessment of biomarkers of BM residual AML cells from DA-treated PDX. DA-resistant PDX cells showed a higher fraction of leukemic LIC subpopulations, thus demonstrating a significant survival advantage relative to the bulk AML, consistent with previous reports [26,29]. Additionally, it has been shown that the CD34+ population derived from AML patients is less sensitive to cytarabine [30]. Our study expanded on the previous findings from multiple AML PDX models, including BM2407, AML146, and AML124, that the CD34+ LIC subpopulation was more resistant to DA compared to the CD34− counterpart. Similarly, the LIC subpopulation CLL1+/CD117− was enriched in the residual disease after DA treatment compared to the control subpopulation, CLL1+/CD117+, in multiple AML models. These results suggest that these chemoresistant LIC subpopulations reside in the BM, where they are protected from chemotherapy-induced apoptosis [7]. Because both the CD34+ and CLL1+/CD117− subpopulations demonstrated self-renewal capacity in NSG mice, these results support the notion that LICs contribute to AML disease relapse.

Although CD33 expression was found on the cell surface of approximately 90% of the AML patients [21], the AML PDX models with successful engraftments in this report presented 100% CD33+ leukemic cells. In addition to the CD34+ LIC subpopulation, almost 100% of the CLL1+/CD17− LICs expressed CD33. These results may suggest that CD33+ cells are preferentially selected through in vivo passage; i.e., the CD33+ AML cells contained in the self-renewing LIC subpopulation are more successfully propagated in mice. This notion is also supported by clinical observations [22,31]. It is noteworthy that the FLT3-ITD mutation, which is a well-known marker of poor prognosis in AML patients [23], was maintained at a high rate via the xenotransplantation of BM0407 and BM2407. A marker connoting an even worse prognosis, DNMT3A/FLT3/NPM1 [24], was also highly maintained in the BM0407 model compared to the initial patient profile. These observations further support the correlation between FLT3-ITD mutations and LICs, as recently reported in AML patient samples [32].

One of the key strategies to improve the overall outcomes in AML is to eradicate the chemoresistant LICs upon reduction of the fast proliferating AML blasts by chemotherapy. Research to date has been unclear about the extent to which the GO-associated antileukemic effect is derived from killing the AML LICs other than its cytotoxicity to bulk AML blasts [33]. In the tested AML models, DA doublet reduced bulk AML cells through apoptosis induction and anti-proliferation (data not shown). In mice that received GO treatment but not the control ADC, BM AML blasts showed double-strand DNA breaks and subsequently apoptosis (data not shown), as expected from the treatment of a calicheamicin drug conjugate. These results highly suggest that CD33 target mediated cell killing. Importantly, GO can completely eradicate the DA-resistant residual disease that contains a higher percentage of LIC subpopulations, as confirmed by using an in vivo limiting dilution analysis. These results demonstrate the anti-LIC function of GO against CD33+ AML LICs, when combined with induction chemotherapy, in addition to its antileukemic effect against bulk CD33+ AML. This notion is also supported by a report from Ehninger and colleagues [31]. In a large cohort of AML patient samples, both the CD33 and CD123 markers were highly expressed in the CD34+ fraction of AML, which is presumably the LIC-enriched population. In contrast, the expression levels of CD33 and CD123 in myeloid progenitors of healthy donors were relatively low compared to the AML blasts. The finding that IMGN779, a CD33-ADC, preferentially inhibits LIC colony formation while sparing normal HSCs [34] further supports CD33 as a valid target for AML.

One of the limitations for clinical translation is that all leukemic blasts express CD33 in tested PDXs, whereas clinically about 10% of AML patients are missing CD33 antigen. Higher fraction of CD33+ blasts could enable better response rate to GO/DA therapy in the AML models than in patients. Future translation work should include a large panel of diverse AML PDX models that reflects the nature of AML heterogeneity.

Despite some limitations, these research outcomes provide insight into the clinical benefits seen in AML patients when GO is added to the existing induction therapy [3,33]. Hence, there is a strong rationale to use GO as a companion agent to target the CD33+ LICs and enhance the existing induction therapy for AML treatment.

Authorship Contributions

C. C. Z., A. J. F., S. H., and P. S. provided guidance for overall concepts; C. C. Z., Z. Y., B. P., and A. J. F. designed the experiment; Z. Y., B. P., Q. Z., M. E., C. F., J. L., and N. H. performed experiments; C. C. Z., Z. Y., B. P., S. H., and M. S. performed data analysis and interpretation; C. C. Z. and M. S. wrote the manuscript; C. C. Z., M. S., and P. S. revised the manuscript.

Disclosure of Potential Conflicts of Interest

All authors are current Pfizer employees. No other potential conflicts of interest were disclosed.

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References

[1] van Der Velden VH, te Marvelde JG, Hoogeveen PG, Bernstein ID, Houtsmlller AB, Berger MS, and van Dongen JJ (2001). Targeting of the CD33-calicheamicin immunoconjugate Mylotarg (CMA-676) in acute myeloid
leukemia: in vivo and in vitro saturation and internalization by leukemic and normal myeloid cells. *Blood* **97**, 3197–3204.

[2] Chevallier P, Robillard N, Ayari S, Guillaume T, Delaunay J, Mecinaud F, Avet-Loiseau H, Mohty M, Harousseau JL, and Garand R (2008). Persistence of CD33 expression at relapse in CD33(+) acute myeloid leukemia patients after receiving gemtuzumab ozogamicin in the course of the disease. *Br J Haematol* **143**, 744–746.

[3] Hills RK, Castaigne S, Appelbaum FR, Delaunay J, Petersdorf S, Orbsus M, Estey EH, Dombret H, Chevet S, and Iffrah N (2014). Addition of gemtuzumab ozogamicin to induction chemotherapy in adult patients with acute myeloid leukemia: a meta-analysis of individual patient data from randomised controlled trials. *Lancet Oncol* **15**, 986–996.

[4] Maurillo L, Bucicciio F, Picoicchi A, Del Principe MI, Sarlo C, Di Veroli A, Hills RK, Castaigne S, Appelbaum FR, Delaunay J, Petersdorf S, Othus M, Estey EH, and Walter RB (2013). FLT3 inhibitors for acute myeloid leukemia: a review of their efficacy and mechanisms of resistance. *Expert Opin Investig Drugs* **22**, 1315–1321.

[5] van Rhenen A, Feller N, Kelder A, Westra AH, Rombouts E, Zweegman S, van der Pol MA, Wainvile Q, Ossenkoppele GJ, and Schuurhuis GJ (2005). High disease as biomarker for optimal biologic dosing of ARA-C in patients with acute myeloid leukemia. *Am J Hematol* **90**, 125–131.

[6] Kiyoi H (2015). Flt3 inhibitors: recent advances and problems for clinical application. *Nagoya J Med Sci* **77**, 7–17.

[7] Zhou J and Chng W-J (2014). Identification and targeting leukemia stem cells: the path to the cure for acute myeloid leukemia. *World J Stem Cells* **6**, 473–484.

[8] Tausig DC, Miraki-Moud F, Anjos-Afonso F, Peavey DJ, Allen K, Röder C, Lilligren D, Oakeeree H, Cavenagh J, and Agrawal SG (2008). Anti-CD38 antibody-mediated clearance of human repopulating cells masks the heterogeneity of leukemia-initiating cells. *Blood* **112**, 568–575.

[9] Wunderlich M, Mizukawa B, Chou F-S, Sexton C, Shrestha M, Sauhara J, and Mulloly JC (2013). AML cells are differentially sensitive to chemotherapy treatment in a human xenograft model. *Blood* **121**, e90–e97.

[10] Zhao X, Singh S, Pardoux C, Zhao J, Hui ED, Abo A, and Korver W (2010). Targeting C-type lectin-like molecule-1 for antibody-mediated immunotherapy in acute myeloid leukemia. *Haematologica* **95**, 71–78.

[11] van Rhenen A, van Dongen GM, Kelder A, Rombouts EJ, Feller N, Moshaver B, Walsum MS-V, Zweegman S, Ossenkoppele GJ, and Jan Schuurhuis G (2007). The novel AML stem cell–associated antigen CILL1-1 aids in discrimination between normal and leukemic stem cells. *Blood* **110**, 2659–2666.

[12] Saito Y, Kitamura H, Hikjata A, Tomizawa-Murasawa M, Tanaka S, Takagi S, Yokoyama A (2011). Inhibition of signal transducer and activator of transcription targets for quiescent, chemotherapy-resistant human leukemia stem cells. *Blood* **117**, 1315–1321.

[13] Kiyoi H (2015). Flt3 inhibitors: recent advances and problems for clinical application. *Nagoya J Med Sci* **77**, 7–17.

[14] Zhou J and Chng W-J (2014). Identification and targeting leukemia stem cells: the path to the cure for acute myeloid leukemia. *World J Stem Cells* **6**, 473–484.

[15] Tausig DC, Miraki-Moud F, Anjos-Afonso F, Peavey DJ, Allen K, Röder C, Lilligren D, Oakeeree H, Cavenagh J, and Agrawal SG (2008). Anti-CD38 antibody-mediated clearance of human repopulating cells masks the heterogeneity of leukemia-initiating cells. *Blood* **112**, 568–575.

[16] Wunderlich M, Mizukawa B, Chou F-S, Sexton C, Shrestha M, Sauhara J, and Mulloly JC (2013). AML cells are differentially sensitive to chemotherapy treatment in a human xenograft model. *Blood* **121**, e90–e97.

[17] Zhao X, Singh S, Pardoux C, Zhao J, Hui ED, Abo A, and Korver W (2010). Targeting C-type lectin-like molecule-1 for antibody-mediated immunotherapy in acute myeloid leukemia. *Haematologica* **95**, 71–78.

[18] van Rhenen A, van Dongen GM, Kelder A, Rombouts EJ, Feller N, Moshaver B, Walsum MS-V, Zweegman S, Ossenkoppele GJ, and Jan Schuurhuis G (2007). The novel AML stem cell–associated antigen CILL1-1 aids in discrimination between normal and leukemic stem cells. *Blood* **110**, 2659–2666.

[19] Saito Y, Kitamura H, Hikjata A, Tomizawa-Murasawa M, Tanaka S, Takagi S, Uchida N, Suzuki N, Sone A, and Najima Y (2010). Identification of therapeutic targets for quiescent, chemotherapy-resistant human leukemia stem cells. *Sci Transl Med* **2**, 17ra19–17ra19.

[20] Ikekoe T, Yang J, Nishioka C, Takeuchi A, Phillip Koehler H, and Yokoyama A (2011). Inhibition of signal transducer and activator of transcription 5 by the inhibitor of janus kinases stimulates dormant human leukemia CD34+/CD38− cells and sensitizes them to anti-leukemia agents. *Int J Cancer* **128**, 2317–2325.

[21] Ehninger A, Kramer M, Rollig C, Thiede C, Bornhauser M, von Bonin M, Wermke M, Feldmann A, Bachmann M, and Ehninger G, et al (2014). Distribution and levels of cell surface expression of CD33 and CD123 in acute myeloid leukemia. *Blood* **123**, e218.

[22] Al-Mawali A, Gillis D, and Lewis I (2016). Immunoeprofiling of leukemia stem cells: CD34+/CD38−/CD123+ delineate FLT3-ITD/ITD-negative clones. *J Hematol Oncol* **9**, 61.

[23] Laszlo GS, Estey EH, and Walter RB (2013). The path and future of CD33 as a therapeutic target in acute myeloid leukemia. *Blood* **28**, 143–153.

[24] Bodd P, Kantarjian H, Ravandi F, and Daver N (2017). Emerging molecular and immune therapies in acute myeloid leukemia. *Am J Hematol* **13**, 4–15.