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Antibody-based therapy of acute myeloid leukemia with gemtuzumab ozogamicin

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1. ABSTRACT

Antibodies have created high expectations for effective yet tolerated therapeutics in acute myeloid leukemia (AML). Hitherto the most exploited target is CD33, a myeloid differentiation antigen found on AML blasts in most patients and, perhaps, leukemic stem cells in some. Treatment efforts have focused on conjugated antibodies, particularly gemtuzumab ozogamicin (GO), an anti-CD33 antibody carrying a toxic calicheamicin-γ_1 derivative that, after intracellular hydrolytic release, induces DNA strand breaks, apoptosis, and cell death. Serving as paradigm for this strategy, GO was the first anti-cancer immunoconjugate to obtain regulatory approval in the U.S. While efficacious as monotherapy in acute promyelocytic leukemia (APL), GO alone induces remissions in less than 25-35% of non-APL AML patients. However, emerging data from well controlled trials now indicate that GO improves survival for many non-APL AML patients, supporting the conclusion that CD33 is a clinically relevant target for some disease subsets. It is thus unfortunate that GO has become unavailable in many parts of the world, and the drug's usefulness should be reconsidered and selected patients granted access to this immunoconjugate.

2. INTRODUCTION

In 2012, approximately 13,780 individuals developed acute myeloid leukemia (AML) in the U.S. (1). Despite aggressive therapies, AML remains difficult to treat, and many patients will die as a consequence of treatment failure or complications from either treatment-related toxicities or impaired normal hematopoiesis. With contemporary 5-year relative survival rates of ~55% for patients <45 years of age but only ~5% for those above age 65 (2-6), the need for effective yet better tolerated new therapies is germane, and in this regard, monoclonal antibodies have raised expectations of accomplishing this goal. In fact, AML has served as a paradigm for their therapeutic use because of well-defined cell surface antigens and easy tumor cell accessibility. Remarkably, although AML cells often express aberrant forms or abundances of surface antigens relative to normal blood cells, true leukemia-specific epitopes have yet to be identified. Still, the number of antigens explored for the treatment of AML is rapidly increasing. Thus far, the most exploited is CD33, most notably as target for the immunoconjugate, gemtuzumab ozogamicin (GO). Being the first anti-cancer antibody-drug conjugate to obtain regulatory approval in the U.S., GO has been pivotal for the concept of antibody-based toxin delivery in oncology. In

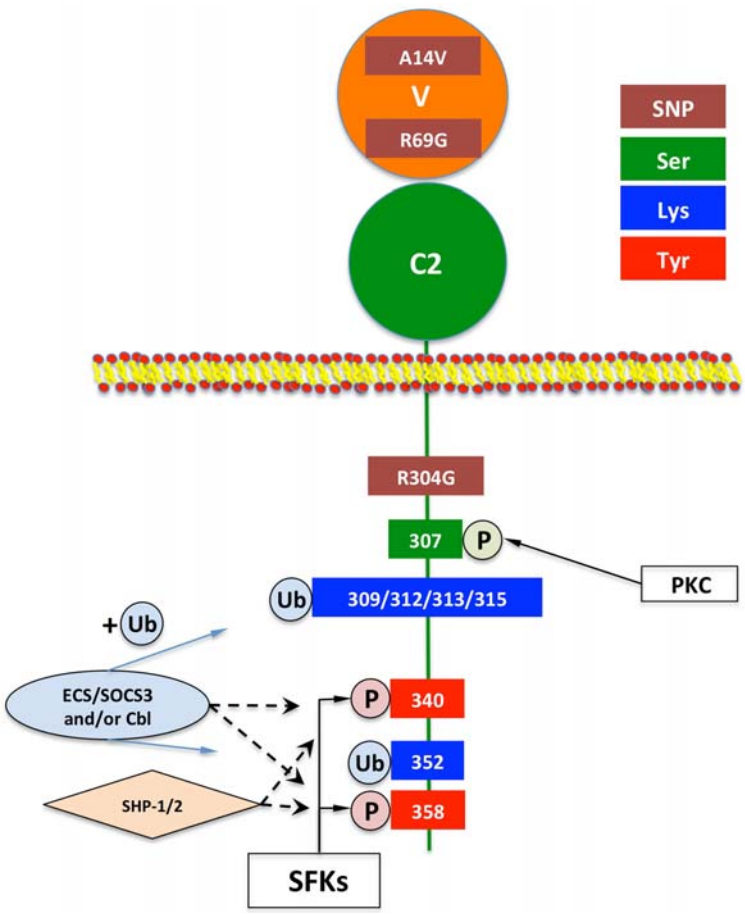

Figure 1. Structure of CD33. Scheme depicting the domain structure of CD33 as well as individual amino acids that have been implicated in phosphorylation or ubiquitylation events or have been identified as residues of relatively frequent non-synonymous single nucleotide polymorphisms (SNPs). Abbreviations: C2, C2-set Ig-like domain; P, phospho-; PKC, protein kinase C; SFKs, Src-family kinases; Ub, ubiquitin; V, V-set Ig-like domain.

this article, we provide an overview of the properties of CD33 that render it suitable for targeting with a toxin-loaded antibody. We review the preclinical development of GO as well as the principles of its mechanisms of action and cellular resistance. We also discuss the clinical experience with GO over the last decade and consider biomarkers that could allow the pre-selection of patients most likely to respond to this drug. Finally, while emerging data on the efficacy of GO support the validity of CD33 as target in AML, we highlight some of the limitations of this approach and describe how these might be overcome in the future.

3. CD33, THE TARGET ANTIGEN

3.1. Physiological properties of CD33

CD33 is a 67kD member of the sialic-acid-binding immunoglobulin-like lectins (Siglecs), a discrete subset of the immunoglobulin (Ig) superfamily molecules (7-10). The human CD33 (Siglec-3) gene, located on chromosome 19q13.3, encodes a single pass, type I transmembrane glycoprotein consisting of an amino-terminal V-set Ig-like domain that mediates sialic-acid recognition, a C2-set Ig-like domain, a transmembrane domain, and an intracellular domain (11-14) (Figure 1). It may be expressed as a homodimer in its physiological state (15). At least in myeloid cell lines – expression in primary cells has not been studied – a shorter isoform lacking exon 2, which encodes the V-set domain, has been identified, but it is unknown whether this splice isoform is expressed on the cell surface (16). The cytoplasmic tail contains 2 conserved tyrosine-based signaling motifs, comprising a membrane-proximal immunoreceptor tyrosine-based inhibitory motif (ITIM) at position 340 and a membrane-distal ITIM-like motif at position 358. Upon phosphorylation, likely by Src family kinases, these tyrosine motifs provide docking sites for the recruitment and activation of the Src homology-2 (SH2) domain-containing tyrosine phosphatases, SHP-1 and SHP-2 (15, 17, 18). While both SHP-1 and SHP-2 are recruited to Y340, Y358 primarily functions to recruit SHP-2. In turn, these tyrosine phosphatases may dephosphorylate CD33 as part of a potential negative feedback control of CD33 signaling (17, 18) or may dephosphorylate and negatively regulate nearby receptors (15). The SH2 domain-containing suppressor of cytokine signaling 3 (SOCS3) can compete

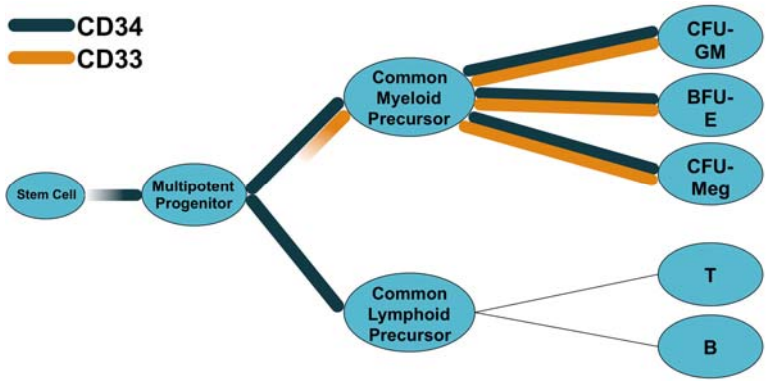

Figure 2. CD33 as myeloid differentiation antigen. Simplified hypothetical model of stem and progenitor cells in the human hematopoietic system, showing expression patterns of CD33 and CD34. This figure was initially published in *Blood* (58). Reproduced with permission from the American Society of Hematology.

with SHP-1/2 for binding to phosphorylated CD33, leading to recruitment of the ECS (Elongin B/C-Cul2/Cul5-SOCS-box protein) E3 ubiquitin ligase complex and concomitant accelerated proteosomal degradation of both CD33 and SOCS3 (19). Indeed, CD33 can become ubiquitylated on several lysine residues located in the cytoplasmic domain. CD33 mono-ubiquitylation or poly-ubiquitylation, which requires intact ITIMs and is enhanced by tyrosine phosphorylation, involves both the lysine cluster around amino acid residues 309-315 and the 352 residue, whereas the first 2 intracellular lysines (residues 283 and 288) contribute little, if at all, to overall ubiquitylation of CD33 (20). Besides SOCS3, the Cbl family of E3 ubiquitin ligases can also bind to CD33 in an ITIM-dependent manner, and ubiquitylation of CD33 by Cbl proteins has been demonstrated experimentally (20).

In addition to tyrosine phosphorylation, CD33 is also rapidly phosphorylated on serine residues as a consequence of protein kinase C activation, with S307 being the strongest putative phosphorylation site. It has been speculated that this may occur as a consequence of cytokine signaling and may regulate its sialic acid-dependent binding activity, but the biological significance of serine phosphorylation of CD33 has not been elucidated in detail (21). Equally little is known about downstream signaling events, although cross-linking of CD33 can induce tyrosine phosphorylation of the proto-oncogenes Cbl and Vav in normal monocytes (22). Likewise, several signaling intermediates (Cbl, Vav, Syk, CrkL, and Plc-γ1) have been shown to form complexes with CD33, at least upon pharmacological tyrosine phosphorylation (22, 23), but the physiological significance of these interactions is unknown.

CD33 exhibits a high degree of sequence similarity with 9 other Siglecs that, together, encompass the rapidly evolving subset of "CD33-related Siglecs" (8). These are mainly expressed on leukocytes in a cell type-specific manner. In healthy individuals, CD33 is primarily found on multipotent myeloid precursors, unipotent colony-forming cells, and maturing granulocytes and monocytes but not outside the hematopoietic system; it is down-regulated to low levels on peripheral granulocytes and resident macrophages while it is retained on circulating monocytes as well as dendritic cells (24-28). Besides expression in the myeloid cell lineage, CD33 may be found on subsets of B lymphocytes and activated human T and natural killer cells (16, 29-34). *In vitro* studies of normal bone marrow indicated that CD33 is not expressed on pluripotent hematopoietic stem cells (26, 27, 35) (Figure 2). Consistently, clinical studies demonstrated delayed but durable multilineage engraftment after transplantation of CD33-depleted autografts in patients with AML (36, 37), providing further evidence that normal hematopoietic stem cells lack CD33. The putative promoter sequence of CD33 contains a critical PU.1 site (38) but the regulation of CD33 expression has so far not been studied in detail; nevertheless, down-regulation of CD33 has been observed on monocytes by activation via T-cell contact, Fcγ receptor cross-linking, or pharmacological stimulation with phorbol myristate acetate or lipopolysaccharide (39).

The physiological function of CD33 is poorly understood. Similar to other CD33-related Siglecs, sialic acid-dependent cell adhesion with preference for α2-6 over α2-3 sialyllactosamines has been demonstrated (13, 17, 40). The adhesive properties of CD33 are modulated by α1-3 linked fucose ("fucosylation"), which reduces binding to α2-3 sialyllactosamines (40), as well as by endogenous sialoglycoconjugates that are present on the cell surface as *cis* ligands (13). Intrinsically, CD33-mediated cell adhesion is regulated by the proximal ITIM motif (13, 17) as well as glycosylation of the extracellular domains; in fact, mutation of a single N-linked glycosylation site in the V-set Ig-like domain can unmask CD33's ligand binding function (41). Because of the ITIM and ITIM-like motifs, CD33 is thought to function as an inhibitory receptor by reducing the activity of tyrosine kinase-driven signaling pathways (10). In support of this notion, early studies demonstrated that cross-linking of CD33 with CD64 (FCGR1A, the high-affinity Fcγ receptor 1a), limits CD64-mediated tyrosine phosphorylation and Ca^{++} mobilization through SHP-1 (15, 18). Increasing evidence suggests that the primary function of CD33-related Siglecs may involve dampening of host immune responses and setting of appropriate activation

thresholds for the regulation of cellular growth, survival, and the production of soluble mediators (9). Consistently, CD33 constitutively suppresses the production of several pro-inflammatory cytokines (IL-1β, TNF-α, and IL-8) by human monocytes in a sialic-acid ligand-dependent and SOCS3-dependent manner (39). Conversely, reduction of cell surface CD33, or interruption of sialic acid binding, leads to activation of p38 mitogen-activated protein kinase (MAPK) and enhances cytokine secretion (39). Likewise, SOCS3 activity reduces CD33-mediated repression of cytokine signaling and enhances cytokine-induced cellular proliferation (19). On the other hand, antibody-engagement of CD33, along with CD33 phosphorylation and recruitment of SHP-1, reduces the syntheses of pro-inflammatory cytokines (TNF- α, IL-6, IL-1β) and chemokines (RANTES, MCP-1, IL-8), in macrophages *in vitro* (42).

While limited, emerging data suggest a role of CD33 in the pathophysiology of several human diseases. Specifically, CD33 expression was found to be significantly reduced on monocytes of patients with type 2 diabetes relative to healthy individuals while secretion of several cytokines (TNF-α, IL-8, IL-12p70) was increased; consistently, high glucose conditions *in vitro* decreased CD33 transcription and protein expression, whereas TNF-α secretion and SOCS3 expression were increased, suggesting a role of CD33 in the generation of the pro-inflammatory milieu characteristic of diabetes (43). Furthermore, although no genetic disorders have been associated so far with mutations in CD33, a single nucleotide polymorphism (SNP) within the CD33 gene (rs3865444) has been associated with the development of Alzheimer's disease (44-46).

3.2. CD33 expression and internalization in AML

Consistent with its physiological expression as myeloid differentiation antigen, 85-90% of adult and pediatric AML patients are considered to have $CD33^+$ disease, defined as the presence of CD33 on greater than 20-25% of the leukemic blasts (25, 47). CD33 is not a highly abundant antigen: quantitative flow cytometry studies estimated that AML blasts display an average of ~10^4 (range: $1x10^3 – 5x10^4$) CD33 molecules per cell (28, 48), and expression is typically even lower in immature (e.g. $CD34^+/CD38^-/CD123^+$) cell subsets (49). From a drug development perspective, an important aspect of CD33 is its internalization when engaged with antibodies (23, 50-56). Mechanistic studies indicate that endocytosis of CD33/antibody complexes is largely limited and determined by the intracellular domain of CD33, while the extracellular and transmembrane domains play a minor role (23, 56). Forced tyrosine phosphorylation enhances the uptake of anti-CD33 antibodies, as does depletion of SHP-1 and SHP-2, at least in some cell lines, consistent with a role of tyrosine phosphorylation as regulator of this process (23). Consistently, disruption of the ITIMs by point mutations prevents optimal internalization of antibody-bound CD33 (56) although some internalization of CD33 occurs in an ITIM-independent manner. Furthermore, ubiquitylation of CD33 decreases CD33 cell surface abundance and increases the rate of CD33 internalization (20). Importantly, compared to antigens such as the transferrin receptor, the internalization process of CD33 is relatively slow (56). Together, the low expression of CD33 and the slow internalization of CD33/antibody complexes leads to relatively limited CD33-mediated drug uptake per unit of time; consequently, for an anti-CD33 antibody-drug conjugate to be most successful, a highly potent toxin will be required.

3.3. CD33 as a potential AML stem cell-associated antigen

It has long been recognized that AML encompasses functionally diverse cells, and disease origination from a leukemic stem cell was first suspected many decades ago (57). Despite intense efforts, however, the cellular origin of AML remains unclear, with ongoing dispute as to whether these leukemias arise from transformed hematopoietic stem cells or emerge as a result of genetic events occurring in more mature progenitor cells (57-62). Regardless of this controversy, the impetus to pursue CD33 as therapeutic target emanated not from the fact that blasts of the vast majority of AML patients express CD33 but from the early notion that some AMLs may predominantly or entirely involve committed $CD33^+$ myeloid precursors, suggesting that this antigen could serve to eradicate underlying malignant stem cells in such leukemias (58). Specifically, classic studies on X chromosome inactivation patterns showed clonal dominance in multiple cell lineages (granulocytes, monocytes, erythrocytes, platelets, and occasionally B lymphocytes) in some leukemias, reflecting origination and expansion at the level of pluripotent $CD33^-$ hematopoietic stem cells. In others, clonal dominance was limited to granulocytes and monocytes, suggesting that expansion of the malignant clone could occur at the committed $CD33^+$ myeloid precursor cell stage (63, 64); an example for the latter may be acute promyelocytic leukemia (APL), as small studies indicate that this disease is mainly expressed in granulocytes/monocytes and predominantly involves $CD33^+$ precursors (65). In these "mature" leukemias, it was hypothesized that $CD33^-$ precursors would be predominantly or completely normal. To test this assumption, $CD33^+$ cells were removed *in vitro* via CD33-directed complement-mediated lysis or fluorescence-activated cell sorting in a small number of patients with such leukemias and the remaining $CD33^-$ cells were placed in long-term culture together with irradiated allogeneic stroma cells (66, 67). Over time, $CD33^-$ precursors from some patients indeed generated colony-forming cells with X chromosome inactivation patterns consistent with predominantly non-clonal hematopoiesis (66, 67). These seminal observations provided the scientific basis for the development and clinical testing of CD33-targeted therapy as a stem cell-directed treatment in a subset of AMLs.

4. GEMTUZUMAB OZOGAMICIN (GO)

4.1. Rationale for use of antibody-drug conjugate to target CD33

Crosslinking of CD33 on AML cells *in vitro* can inhibit the proliferation of these cells and activate a process leading to apoptotic cell death (68, 69). First attempts to

exploit CD33 for targeted AML therapy in the clinic were undertaken with an unconjugated murine anti-CD33 antibody (M195). Although saturation of CD33 binding sites was observed with doses around 5 mg/m^2, however, only some patients had transient decreases in peripheral blast counts at this or higher doses (50). Subsequent studies employed a humanized IgG_1 construct of M195, lintuzumab (HuM195; SGN-33), which had >8-fold higher binding avidity than the parent antibody and, unlike M195, demonstrated antibody-dependent cell-mediated cytotoxicity (51, 70). Limited studies pointed towards some activity in APL when used in combination with all-trans retinoic acid (ATRA) in patients in morphological complete remission (CR) (71). On the other hand, lintuzumab had very modest activity as a single agent in overt non-APL AML, with infrequent achievement of CR or partial remission (PR) only amongst patients with relatively low tumor burden even at supra-saturating antibody doses (12-36 mg/m^2 per day for 4 days x 2 courses) that fully blocked CD33 binding sites throughout a 4-week period (72, 73). Higher doses of lintuzumab (1.5-8 mg/kg/week for 5 weeks, followed by every other week treatment for those who experienced clinical benefit) appeared somewhat more efficacious when investigated in patients with $CD33^+$ myeloid malignancies: among the 17 patients with AML, 7 had an objective response (4 morphologic CRs, 2 partial remissions (PRs), and 1 morphologic leukemia-free state) with a median duration of therapy of 25.1 (range, 4.1-57.1) weeks (74).

Two randomized trials have tested lintuzumab together with conventional chemotherapy. In the first, 191 patients with relapsed/refractory AML were randomly assigned to receive mitoxantrone, etoposide, and cytarabine with or without lintuzumab (12 mg/m^2). Addition of lintuzumab was associated with an insignificantly higher overall response rate (ORR; CR + CR with incomplete platelet recovery [CRp]: 36% vs. 28%, p=0.28) but unchanged overall survival (OS) (75). In the second, 211 patients older than age 60 with untreated AML were randomized to receive low-dose cytarabine (20 mg subcutaneously twice daily for 10 days) with either lintuzumab (600 mg/week for 4 doses in cycle 1 and every other week for 2 doses in subsequent cycles) or placebo in a double-blinded phase 2b study. Again, addition of lintuzumab did not improve OS (76). Ultimately, because of these negative results, the clinical development of lintuzumab was terminated in 2010.

The lack of significant tumor reducing effects of saturating or supra-saturating doses of unconjugated anti-CD33 antibodies in patients with overt non-APL AML indicated that anti-CD33 antibodies would be useful for AML therapy only if they served as a carrier of another biologically active agent. The feasibility of such an approach was suggested by studies with radiolabeled anti-CD33 antibodies showing selective uptake of the radio-immunoconjugate by AML cells and rapid saturation of leukemic blast cells in peripheral blood and bone marrow at intravenous doses of ≥5mg/m^2 (50, 52, 77). While the endocytic property of CD33 proved to be a hurdle for the delivery of radioiodine due radio-immunoconjugate internalization and metabolization and, consequently, relatively short residence times in the marrow (50, 52, 77), it spurred efforts to develop CD33-targeting antibody-drug conjugates carrying a toxic payload.

4.2. Development of GO

The class of toxin selected were the calicheamicins, highly potent and reactive antitumor antibiotics of the enediyne family that were originally isolated from fermentations of the soil microorganism *Micromonospora echinospora* ssp. *calichensis* in a screen for potent DNA damaging agents (78-81). The parent compound, calicheamicin-γ_1^I, has been shown to interact with double-stranded DNA in the minor groove in a relatively sequence-specific manner *in vitro* (82). Following reduction by cellular thiols, the enediyne moiety undergoes rearrangement to form a 1,4-benzenoid diradical that abstracts hydrogens from the phosphodiester backbone of DNA, resulting in single- and double-strand lesions (82, 83) (Figure 3); the latter involve direct double-strand breaks and, as a major lesion, bistranded damage that consists of an abasic site on one strand and a direct strand break on the other (84). This DNA damage elicits a strong cellular response with cell cycle arrest in the G_2/M phase followed by either DNA repair or, if damage is overwhelming, apoptosis and cell death. While the response to the initial DNA damage remains incompletely understood, calicheamicin-induced double-strand breaks activate DNA repair through activation of ATM/ATR and DNA-dependent protein kinase (DNA-PK) (85, 86). In turn, ATM activation leads to activation of Chk1/2 and G_2/M cell cycle arrest (85, 87). DNA-PK phosphorylates H2AX in rapid response to DSBs, a step that is required for subsequent recruitment of DNA damage repair proteins (88). Consistently, cells defective in ATM or DNA-PK are hypersensitive to calicheamicins (83, 89), as are cells deficient in the ERCC2/XRD gene, which is involved in the nucleotide excision repair pathway (90), supporting the notion that the extent of DNA damage and damage repair is central for the toxic effects of calicheamicins. Some experimental studies have suggested that calicheamicin-induced cytotoxicity could involve non-apoptotic (i.e. necrotic) pathways, e.g. through activation of poly(ADP-ribose) polymerase 1 (PARP1) and exhaustion of NAD^+ levels (91). However, the mitochondrial pathway of apoptosis appears to be predominantly utilized during calicheamicin-induced cell death, which may be triggered in a p53-independent and death receptor/FADD-independent manner via activation of mitochondrial permeability transition, cytochrome c release, involvement of pro-apoptotic Bcl-2 family proteins (e.g. Bax and Bak), and activation of caspases (92, 93). In line with this cytotoxic mechanism, microarray studies in yeast indicate that calicheamicin-γ_1^I alters the expression of genes involved in chromatin arrangement, DNA repair and/or oxidative damage, DNA synthesis and cell cycle checkpoint control but also a variety of metabolic, biosynthetic, and stress response genes, as well as ribosomal proteins (94).

Experiments with free and antibody-bound calicheamicin analogues determined the structure-activity

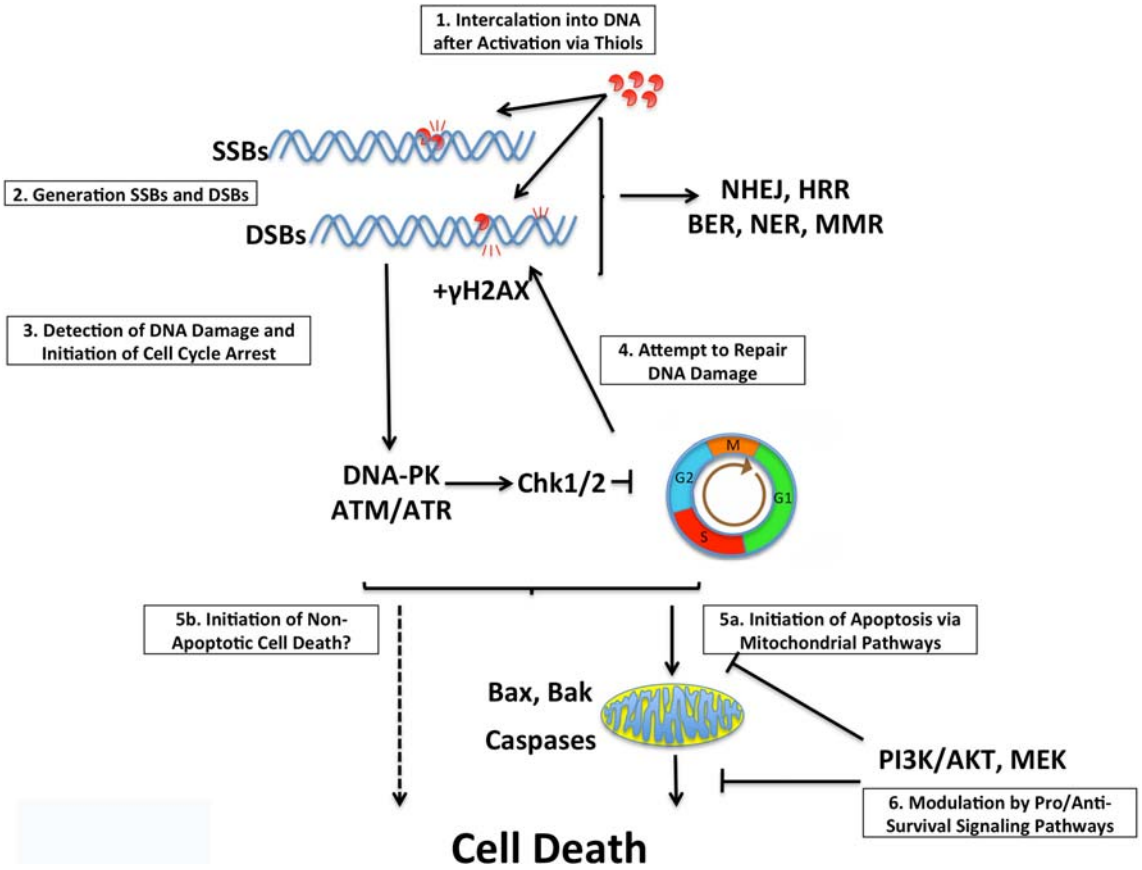

Figure 3. Calicheamicin-induced cytotoxicity. Scheme depicting the presumed mechanism of cytotoxicity of calicheamicins in AML cells. Abbreviations: BER, base excision repair; γH2AX, phosphorylated H2AX; DSBs, double-strand breaks; HRR, homologous recombinational repair; NER, nucleotide excision repair; NHEJ, non-homologous end joining; MMR, mismatch repair; SSBs, single-strand breaks

profile of a series of these toxins (95). For conjugation via periodate oxidized carbohydrates contained on the anti-MUC1 antibody, CTM01, thiol hydrazide derivatives were prepared by displacement at the methyltrisulfide moiety of the parent analogues. This process results in a "carbohydrate conjugate" capable of releasing active drug both by hydrolysis of the hydrazone bond at low pH as well as by reduction of the disulfide bond. Compared with calicheamicin-γ_1^I, analogues that were missing the rhamnose at the end of the DNA binding region were found ineffective as conjugates *in* vivo; in contrast, 2 analogues (calicheamicin-α3I and N-acetyl-calicheamicin-γ1I) in which the DNA binding region was intact yet the amino sugar was either eliminated or modified showed a clear therapeutic advantage over calicheamicin-γ1I. Addition of methyl groups as steric bulk adjacent to the disulfide in the linker resulted in enhanced anti-tumor activity and an improved therapeutic window, likely because of increased stability of the linker in the serum. Together, these early studies identified the N-acetyl-calicheamicin-γ_1^I dimethyl hydrazide derivative as having an optimal therapeutic window when conjugated to an antibody (95). Of note, although the potency of this hydrazide is 2-8 fold less than that of the corresponding parent compounds, it remains 100-1,000-fold more potent than clinically used anti-cancer agents. While not suited as free drug due to a narrow therapeutic window, this potency renders a calicheamicin a good candidate as toxin for antibody-based therapeutics (78).

During early development, a murine antibody (P67.6) recognizing the V-set Ig-like domain of CD33 (96) was conjugated to the N-acetyl-calicheamicin-γ_1^I dimethyl hydrazide derivative ("carbohydrate conjugate") as well as to a N-acetyl-calicheamicin-γ_1^I dimethyl acid, N-hydroxysuccinimide ester; conjugation of the latter occurred via lysine residues of the antibody, resulting in an "amide conjugate" stable to hydrolysis (97). While inclusion of the hydrazone was not necessary for anti-tumor activity of the anti-MUC1 antibody (98), only the carbohydrate conjugate of P67.6 showed good potency and selectivity against $CD33^+$ human AML cells *in vitro* and in xenograft models, demonstrating the importance of rapid release of the toxic moiety from its conjugated state under acidic conditions such as those in lysosomal vesicles for anti-AML activity (97). Subsequently, P67.6 was humanized by grafting complementarity-determining regions into a human IgG_4 kappa framework (hP67.6) to minimize immunogenicity, and then conjugated with the calicheamicin derivative via an acid-labile hybrid 4-(4'-

Table 1. Cellular parameters implicated in GO efficacy

Factor	Comment
Uptake of CD33/GO complexes	
Receptor-mediated uptake	
• CD33 expression levels	Quantitative relationship between CD33 expression and GO efficacy in engineered cell lines; expression levels associated with cytogenetic risk of AML and CD33 SNPs
• CD33 saturation	*In vitro* evidence linking reduced CD33 saturation to reduced GO cytotoxicity
• CD33 internalization	Relatively slow process, controlled by intracellular tyrosine motifs and likely tyrosine phosphorylation and ubiquitylation status of CD33
• Re-expression of CD33 binding sites	Surface CD33 levels return to pretreatment levels within 72 hours after CD33 antibody administration; could contribute to amount of internalized GO, in particular if given in fractionated doses.
Non-receptor mediated uptake	Suggested by experimental studies with $CD33^-$ cell lines (clinical role unknown)
Intracellular trafficking of GO	**Hypothetical**
Activation of GO	Low pH in lysosomes required for release of calicheamicin-γ_1^I moiety from antibody
Extrusion of GO	
• ABC family of drug transporters	Established role of P-glycoprotein and MRP1; role of other transporters unknown
Induction of cytotoxicity	
• Generation of SS- and DS-DNA breaks	Hypersensitivity of cell lines with defects in DNA repair to calicheamicins
• Mitochondrial pathways of apoptosis	Good experimental evidence for role of pro- and anti-apoptotic Bcl-2 protein family members
• Other downstream pro- or anti-apoptotic signaling pathways	Not examined in detail but MEK1/2 and AKT signaling may confer relative resistance
• Cell cycle status	Limited *in vitro* data suggesting that resting cells are relatively less susceptible to GO

Abbreviations: DS, double-stranded; GO, gemtuzumab ozogamicin; SNP, single-nucleotide polymorphism; SS, single-stranded Modified from a table that was initially published in *Blood* (58). Reproduced with permission from The American Society of Hematology

acetylphenoxy)butanoic acid linker to yield GO (99) (Figure 4). Of note, hP67.6 contains an IgG_4 core-hinge mutation that protects the therapeutic from Fab-arm exchange with endogenous human IgG_4 and thereby provides stabilization of the drug (100). However, only about 50% of the antibody is linked to calicheamicin-γ_1 moieties, with an average loading of 4-6 molecules of the calicheamicin-γ_1 derivate per antibody, while the remaining antibody is unconjugated (101).

5. PRECLINICAL OBSERVATIONS WITH GO

Initial preclinical studies showed that GO exerted selective cytotoxicity against $CD33^+$ AML cells, effectively inhibiting colony-forming cells in pediatric and adult AML specimens *in vitro* whereas a control immunoconjugate did not, and caused regression of $CD33^+$ AML cell line xenografts in athymic mice (99). Subsequent *in vitro* studies have confirmed these findings and provided insight into the cellular characteristics that are relevant for the clinical GO efficacy (Table 1).

In contrast to other anti-CD33 antibodies (68, 69), both P67.6 and hP67.6 are largely non-toxic against $CD33^+$ AML cell lines and human AML specimens (87, 97, 102). It is thus thought that the antibody component primarily functions as a carrier to facilitate cellular uptake of the calicheamicin-γ_1^I derivative into $CD33^+$ cells (Figure 5). The contribution of non-receptor mediated endocytic drug uptake, a possibility suggested by limited *in vitro* studies with $CD33^-$ acute lymphoblastic leukemia cell lines (103), for clinical GO efficacy is unknown. Internalized CD33/GO complexes are routed to lysosomes, where the toxic moiety is presumably released (54, 104). The free calicheamicin-γ_1^I derivative can then enter the nucleus and initiate DNA damage. This putative mechanism of action implies a critical role for the intracellular accumulation of the calicheamicin-γ_1^I derivative as well as the cellular response to the toxin's DNA damaging effect for GO-induced cytotoxicity (Table 1). Conceptually, the intracellular load of activated calicheamicin-γ_1^I is impacted by the amount of GO uptake, the efficacy of toxin release from the antibody and subsequent activation via cellular thiols, as well as toxin inactivation/metabolism or expulsion. However, while induction of DNA damage appears to be a prerequisite for GO-induced cytotoxicity (105, 106), it is not sufficient, indicating that the toxicity of the calicheamicin-γ_1^I moiety is modulated by the cell's ability to repair DNA damage and the activity of downstream pro- and anti-apoptotic pathways. Overall, the sensitivity to the toxic moiety varies over 100,000-fold between individual primary AML cells samples (107), an observation that emphasizes the importance of patient-specific factors for the clinical efficacy of GO.

Several patient-specific factors have been identified: most importantly, studies have repeatedly shown that drug efflux mediated by members of the adenosine triphosphate (ATP) binding cassette (ABC) superfamily of proteins, predominantly P-glycoprotein (ABCB1) and to a lesser degree multidrug resistance protein 1 (MRP1; ABCC1) but not breast cancer resistance protein (BCRP; ABCG2), mediate resistance to GO; conversely, inhibition of drug efflux effectively increases GO-induced cytotoxicity *in vitro* (87, 108-113). Taken together, these investigations have identified drug efflux as a major determinant of GO's anti-AML activity. Experimental studies also revealed a striking, quantitative relationship between CD33 expression and GO efficacy in engineered human AML cell lines and demonstrated the requirement of GO/CD33 complex internalization for GO-induced cytotoxicity (56). Thus, the amount of GO uptake is a limiting factor for GO efficacy. In support of this notion, CD33 expression levels directly correlate with the *in vitro* sensitivity of immature AML cell fractions to GO (102, 114). While the role of DNA repair and downstream signaling pathways for GO efficacy has not yet been examined in detail, Bcl-2 family proteins modulate GO cytotoxicity against AML cell lines (110). Furthermore, recent studies found activated PI3K/AKT signaling to be associated with GO resistance *in vitro* in primary AML

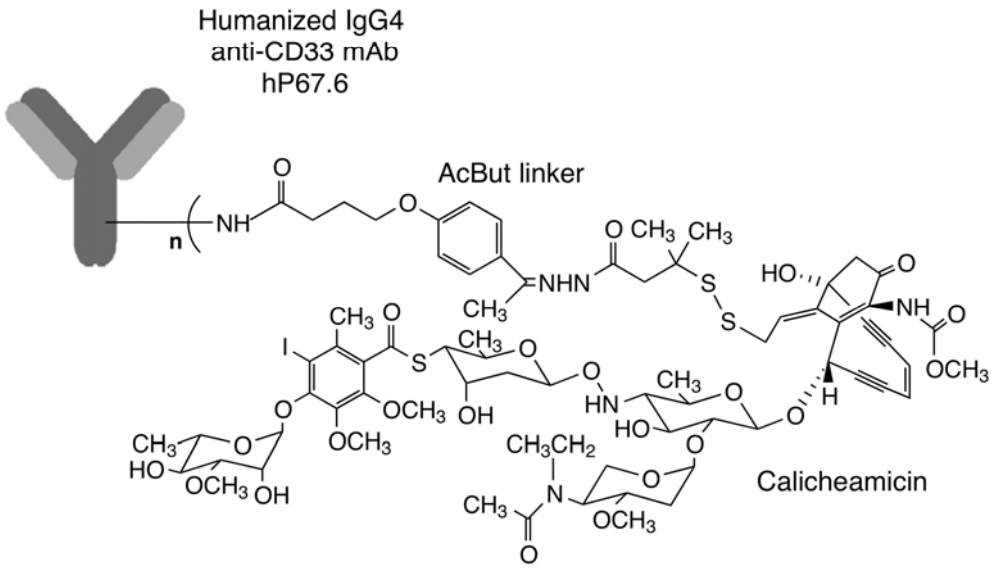

Figure 4. Schematic structure of GO. This figure was initially published in *Current Opinion in Pharmacology* (78). Reproduced with permission from Elsevier.

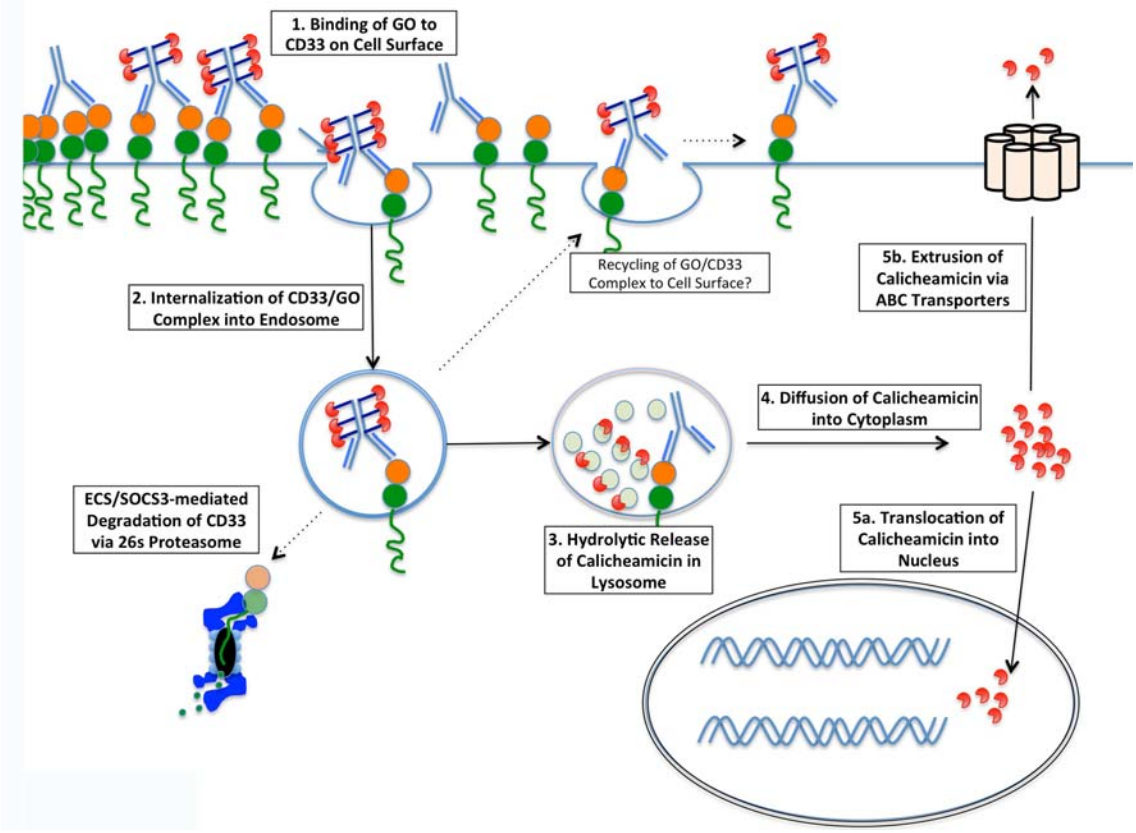

Figure 5. Schemes depicting the presumed mechanism of GO uptake, trafficking, and intracellular release of the calicheamicin-γ_1^I derivative with subsequent translocation into the nucleus or extrusion via ABC transporter proteins.

cells, whereas the investigational AKT inhibitor, MK-2206, sensitizes various human AML cells to GO or free calicheamicin-γ_1^I (106). Likewise, limited data suggest that MEK1/2 activity may be involved in resistance to GO (115). Curiously, the presence of an FLT3/ITD mutation has been associated with increased sensitivity of immature AML cells to GO (114) but, so far, studies have not identified this marker to predict clinical response to GO.

Finally, some studies have suggested Syk expression to be a biomarker of response to GO, and depletion of Syk to result in unresponsiveness to GO (116). However, in our experience, depletion of Syk by lentivirus-mediated siRNA expression did not affect GO cytotoxicity in engineered AML cell lines (R.B.W. unpublished observation).

A number of studies have indicated that the sensitivity of AML cells to GO can be enhanced through the use of other agents. Such chemosensitizing effects have been observed with histone deacetylase inhibitors (117), DNA methyltransferase I inhibitors (116, 118), the farnesyl transferase inhibitor, tipifarnib (119), heat shock protein-90 inhibitors (40), anti-CD45 antibodies (120), as well as mitoxantrone (121). Furthermore, the combination of GO with other conventional chemotherapeutics such as cytarabine, daunorubicin, idarubicin, doxorubicin, etoposide, or 6-mercaptopurine has additive cytotoxic effects *in vitro*, whereas methotrexate and vincristine may antagonize the effects of GO (121, 122). Interestingly, no rigorous studies have attempted to define the optimal timing when GO should be given with other agents. The timing, especially with respect to the cell cycle status, is likely of some importance, however, in view of data from *in vitro* studies suggesting that resting cells are relatively less susceptible to GO while treatment with granulocyte colony-stimulating factor (G-CSF) sensitizes to GO (103, 123). Finally, an interesting yet unstudied aspect of CD33-targeted immunotherapy is the question whether shorter isoforms lacking the extracellular V-set Ig-like domain and antibody epitope (16) could modulate the efficacy of anti-CD33 antibody-based drugs.

6. CLINICAL PHARMACOLOGY OF GO

Pharmacokinetic studies in rats and monkeys identified the hepatobiliary system as the major excretion pathway for single and repeated doses of GO (101). *In vitro*, numerous metabolites of GO are formed, with biotransformation pathways involving both liver microsomes (oxygenation and demethylation) and the cytosol (acetylation of calicheamicin-γ_1^I derivative) (101). In humans, most of the available information on pharmacokinetics and pharmacodynamics is from adult patients who received 1-2 doses of GO at 9 mg/m^2 given 14-28 days apart on the phase 2 monotherapy trials. A mean maximum plasma concentration of 2.86 ± 1.35 mg/L was measured shortly after the end of the first 2-hour infusion of GO, and near complete saturation of CD33 antigenic sites in the peripheral blood was reached within 3-6 hours (55, 124). The drug elimination half-life proved highly variable, ranging from 72.4 ± 42.0 hours to 45.1 ± 25.2 hours for hP67.6 and calicheamicin, respectively, after the first dose of GO (124). Even 2 weeks after drug administration, circulating levels of GO are sufficient to partially saturate CD33 sites in the peripheral blood (55). Consistently, the area under the curve (AUC) was found to be higher in the second dosing period, with a 94% increase relative to the first administration (239±196 vs. 123±105 mg x h/L) and a corresponding decrease in clearance (0.132±0.153 vs. 0.265±0.229 L/h), perhaps related to the decreased peripheral blood CD33 antigen load at the time of second dosing; the latter would imply that CD33 antigen binding impacts pharmacokinetics as the principal means of elimination of hP67.6 from the plasma. After the second dose of GO, maximum serum concentrations were higher (3.67±1.30 mg/L) and drug elimination longer (half-lives of 93.7±67.4 hours and 61.1±45.4 hours for hP67.6 and calicheamicin-γ_1^I, respectively) than after the first dose. The nearly parallel time-concentration plasma curves for hP67.6 and total calicheamicin-γ_1^I in most patients suggested that the toxic moiety remained largely bound to the antibody; indeed, free calicheamicin-γ_1^I could only be measured for a relatively short time following the end of drug infusion (124). Of note, although inter-patient variability is high, the fundamental pharmacokinetics do not appear to differ in relationship to age, gender, or ethnicity (124-127), and, among a set of 59 patients, no relationship was found between the pharmacokinetic parameters and response (124).

7. CLINICAL EFFICACY OF GO

7.1. Early clinical studies and accelerated approval of GO

A phase 1 study among 40 adults with relapsed/refractory CD33$^+$ AML who received up to 3 doses of GO in 2-week intervals found morphologic elimination of leukemia in 8 (20%) patients, with 3 (7.5%) achieving CR and 2 (5%) achieving CRp. As dose-limiting non-hematologic toxicity was not reached, the highest dose level of 9 mg/m^2, which provided almost complete saturation of CD33 binding sites, was chosen for further study (128). Three open-label, multicenter single-arm phase 2 trials then evaluated GO in a larger cohort of adults with CD33$^+$ *de novo* AML in first relapse. An interim analysis on 142 patients (median age: 61 [range: 22-84] years) who typically received 2 doses of GO 14 days apart showed an ORR of 29.6% (CR: 16.2%; CRp: 13.4%) (129). Presented with these results, the Oncology Drugs Advisory Committee (ODAC) concluded at the March 14, 2000 meeting that this drug provided a useful therapeutic option in patients >60 years of age with CD33$^+$ AML in first relapse who would not be candidates for standard cytotoxic chemotherapy (101). The U.S. Food and Drug Administration (FDA) accepted this recommendation, and GO was given accelerated marketing approval on May 17, 2000 for this indication. Of note, the approval regulations not only required the sponsor to complete the original phase 2 studies in relapsed AML but also mandated the conduct of randomized, controlled trials to confirm the clinical benefit of GO when added to conventional chemotherapy in patients with *de novo* AML (101).

7.2. Subsequent clinical trials and discontinuation of commercial availability of GO

The final report of the 3 pivotal phase 2 trials on 277 patients confirmed the early results in relapsed AML, with 35 (12.6%) and 36 (13.0%) patients achieving CR and CRp, respectively, for an ORR of 25.6%, although remission durations were relatively short (130). Numerous studies have subsequently investigated GO in various clinical situations. As results from most of these trials have been reviewed previously in detail (131-137), we will only

Table 2. Phase 3 studies of GO in newly diagnosed non-APL AML

Study	Reference	Disease	N	Age (median)	Treatment	Results
MRC/NCRI AML15	(146)	AML	1,113	0-71 (49)	± GO (3 mg/m^2) on day 1 of the first of two induction courses with either ADE, DA, or FLAG-IDA	No difference in ORR, TRM, relapse, or survival. Improved 5-year OS for favorable-risk subgroup (79% vs. 51%; p=0.0003) with GO; predicted 10% OS benefit for ~70% of patients with intermediate-risk disease
ALFA 0701	(145)	AML	278	59-66 (62)	± GO (3 mg/m^2) on days 1, 4, and 7 of DA induction and day 1 of each of 2 courses of DA consolidation	No difference in ORR or mortality. Improved 2-year EFS (40.8% vs. 17.1%, p=0.0003), DFS (50.3% vs. 22.7%, p=0.0003) and OS (53.2% vs. 41.9%, p=0.037) with GO. Survival benefit seen in favorable/intermediate- but not adverse-risk disease
GOELAMS AML 2006 IR	(147)	AML (intermediate risk)	238	18-60 (50)	± GO (6 mg/m^2) on day 1 with DA induction and MA consolidation	No difference in ORR, TRM, or 3-year EFS. Improved EFS with GO in patients who did not undergo allogeneic HCT (53.7% vs. 27%, p=0.0308)
MRC/NCRI AML16	(148)	AML, high-risk MDS	1,115	51-84 (67)	± GO (3 mg/m^2) on day 1 of the first of two induction courses with either DA or DCLo	No difference in TRM; trend towards reduced risk of persistent disease with GO (17% vs. 21%, p=0.06). Reduced 3-year relapse risk (68% vs. 76%; p=0.007) and superior DFS (21% vs. 16%; p=0.04) and OS (25% vs. 20%; p=0.05) with GO
SWOG S0106	(149, 150)	AML	596	18-60 (47)	± GO (6 mg/m^2) on day 4 of the first of up to two induction courses with DA*	Increased TRM in GO arm (5.7% vs. 1.4%; p=0.01). No difference in ORR, DFS, or OS. Possible trend towards improved OS in favorable-risk subgroup with GO (hazard ratio: 0.49 [0.12-2.04])

Abbreviations: ADE, cytarabine/daunorubicin/etoposide; DA, daunorubicin/cytarabine; DClo, daunorubicin/clofarabine; DFS, disease-free survival; EFS, event-free survival; FLAG-Ida, fludarabine/cytarabine/G-CSF/idarubicin; HCT, hematopoietic cell transplantation; MA, mitoxantrone/cytarabine; ORR, overall response rate (CR+CRi); OS, overall survival; TRM, treatment-related mortality, *Daunorubicin was used at 60 mg/m^2/day in the control (-GO) arm and 45 mg/m^2/day in the GO arm. This table was initially published in *Blood* (58). Reproduced with permission from The American Society of Hematology.

highlight those trials that are most informative regarding the potential clinical utility of GO.

7.2.1. Treatment of APL

GO is likely most effective in APL. For example, single agent GO resulted in molecular CR in 9 of 11 patients with molecularly relapsed APL tested after 2 doses and in 13 of 13 patients tested after the 3rd dose (138). Additional case reports corroborate the notion of very high efficacy of GO in the setting of minimal residual disease in APL, including patients with very advanced disease (139, 140). Furthermore, phase 2 trials suggest that GO can substitute for anthracycline therapy even in high-risk disease, and add benefit to therapy with ATRA (141, 142).

7.2.2. Treatment of pediatric non-APL AML

So far, only a few clinical studies have explored GO in childhood AML, primarily in patients with relapsed/refractory disease, and GO has no established role in this patient population. However, a large randomized trial testing the addition of GO to induction chemotherapy was led by the Children's Oncology Group (AAML0531) and has recently completed accrual of >1,000 participants but preliminary outcome results are expected for 2013 at the earliest.

7.2.3. Treatment of adult non-APL AML

Several phase 2 studies investigated GO monotherapy in unselected patients with newly diagnosed and/or relapsed/refractory non-APL AML. While these studies confirmed single agent activity of GO, the ORRs have typically not exceeded 25-35% and were occasionally quite disappointing, particularly in heavily pretreated patients. Of note, most of these studies followed a 2-weekly schedule of GO administration. Less well explored is the use of GO given at shorter intervals. However, the ALFA group has relatively recently reported the use of GO in fractionated, lower doses (3 mg/m^2 on days 1,4, and 7) with promising efficacy and acceptable toxicity (143-145), but no direct, controlled comparisons with the traditional administration schedule have been made.

A wealth of studies have explored GO with other therapeutics or chemosensitizers and overall yielded mixed results. As most were small, single arm trials, no firm conclusions can be drawn from the majority of these studies as to the efficacy of GO relative to other drugs, or whether the addition of GO provided any benefit over what would have been achieved with the other therapeutics alone. Recently, however, several well controlled large studies have been conducted that congruently show that GO improves survival in a significant and relatively well defined subset of patients with newly diagnosed non-APL AML when added to conventional chemotherapy (58) (Table 2). In the MRC/NCRI AML15 trial, 1,113 predominantly adult patients were randomized to receive a single dose of GO (3 mg/m^2) on day 1 of the first of two induction courses with one of three induction regimens (cytarabine/daunorubicin/etoposide, daunorubicin/cytarabine, fludarabine/cytarabine/G-CSF/idarubicin). While adding GO did not affect ORRs or survival across the entire study cohort, subgroup analyses showed that addition of GO improved OS at 5 years in patients with favorable cytogenetics (79% vs. 51%; p=0.0003) but not in those with unfavorable cytogenetics (8% vs. 11%; p=0.4). There was also a survival benefit for some intermediate-risk patients, as indicated by an internally validated index using cytogenetics, age, and performance status, which predicted that ~70% of these patients had an improved 5-year OS if given GO (146).

In the ALFA 0701 trial, 278 patients with primary AML aged 50-70 years received daunorubicin/cytarabine with or without GO (3 mg/m^2) on days 1, 4, and 7; a second course of

daunorubicin/cytarabine was given for residual disease on day 15. Patients in remission then received 2 courses of daunorubicin/cytarabine with or without GO (3 mg/m^2) on day 1 of each cycle. While there was no statistically significant difference in ORRs and treatment-related mortality (TRM) between the 2 arms, the event-free survival (EFS) at 2 years was significantly superior in the GO arm (40.8% vs. 17.1%, p=0.0003), as was disease-free survival (DFS) (50.3% vs. 22.7%, p=0.0003) and OS (53.2% vs. 41.9%, p=0.037). Subgroup analysis showed that the EFS benefit occurred in patients with favorable/intermediate cytogenetics but not in those with an adverse karyotype (145). In the GOELAMS AML 2006 IR study, adults aged 18-60 years with *de novo* AML and intermediate karyotype received daunorubicin/cytarabine with or without GO (6 mg/m^2) on day 4; GO was also added to consolidation therapy according to the initial randomization (147). Among 238 analyzed patients, there was no difference in ORRs or TRM between the 2 treatment arms. Overall, there was also no statistically significant difference in EFS (GO vs. control: 51% vs. 33%) or OS (53% vs. 46%) at 3 years. However, subgroup analyses showed that in patients who did not undergo allogeneic hematopoietic cell transplantation (HCT), EFS was significantly higher in the GO group (53.7% vs. 27%, p=0.0308). Finally, in the MRC/NCRI AML16 trial, 1,115 adults aged 51-84 years with AML or high-risk myelodysplastic syndromes (MDS; >10% marrow blasts) received either daunorubicin/cytarabine or daunorubicin/clofarabine for 2 cycles with or without GO (3 mg/m^2) on day 1 of the first induction course (148). Similar to the other trials, there was no significant difference in ORRs and TRM between the treatment arms, although the patients receiving GO had a slightly lower likelihood of persistent disease (17% vs. 21%, p=0.06). However, use of GO was associated with reduced relapse risk (at 3 years: 68% vs. 76%; p=0.007) and superior DFS (21% vs. 16%; p=0.04) as well as OS (25% vs. 20%; p=0.05). A meta-analysis of AML15 and AML16 on 2,224 patients showed a significant benefit of GO for risk of relapse (odds ratio [OR]: 0.82 [95% confidence interval: 0.72-0.93], p=0.002) and survival (OR: 0.88 [0.79-0.98], p=0.02); the survival benefit was seen in patients with favorable-risk (OR: 0.47 [0.28-0.77]) and intermediate-risk (OR: 0.84 [0.73-0.97]) but not adverse-risk (OR: 1.02 [0.81-1.27]) disease (148).

The results from these European studies differ from the SWOG trial S0106, which was developed with the drug sponsor to fulfill the post-approval commitment to the FDA. Six hundred thirty-seven patients aged 18-60 years with *de novo* AML were accrued to receive up to 2 cycles of induction chemotherapy with daunorubicin/cytarabine with or without a single dose of GO (6 mg/m^2) on day 4 of the first induction (149). Unlike the European studies in which identical doses of conventional chemotherapeutics were used in both arms, S0106 used a lower daunorubicin dose with GO (45 mg/m^2 vs. 60 mg/m^2). Overall, among the 596 evaluable patients, S0106 showed no difference in response or survival in either induction of post-consolidation with the addition of GO, but a trend towards improved OS was seen in patients with favorable-risk leukemias (hazard ratio: 0.49 [0.12-2.04], although S0106 was not powered to detect important outcome differences in this patient subset. As there was an attempt to achieve equi-toxicity with the lower dose of daunorubicin in the GO arm, the S0106 trial is confounded by the lower dose of daunorubicin administered to patients receiving GO, which might mask a greater benefit of the immunoconjugate. Nonetheless, based on the lack of pre-specified overall improvement in outcome, S0106 was prematurely terminated (150).

In contrast to its use in induction therapy, so far no study has documented a benefit of GO when used in consolidation. ECOG used a high-dose cytarabine-containing post-remission strategy incorporating a single dose of GO (6 mg/m^2) for patients 17-60 years of age in first CR in a randomized phase 3 trial. Among 352 randomized patients, results of intent-to-treat analyses failed to provide any evidence of a benefit of GO in this trial (151). In the HOVON-43 study, 232 patients 60 years or older who achieved a first CR after intensive induction chemotherapy were randomized to 3 cycles of GO (6 mg/m^2 every 4 weeks) or no post-remission therapy; again, no differences in relapse probability, survival, or non-relapse mortality were noted (152).

7.2.4. Treatment of the elderly or unfit with non-APL AML

In contrast to the situation when GO is added to intensive chemotherapy, its benefit when combined with low-dose chemotherapy is perhaps more limited. This is suggested by a recent trial conducted by the MRC/NCRI, in which 495 patients were randomized to receive low-dose cytarabine with or without GO at 5 mg/m^2 on day 1 of every course; in this study, GO almost doubled the CR rate (30% vs. 17%, p=0.006) but did not improve the 12-month overall survival (153).

7.2.5. Withdrawal of GO from commercial market

Following discussions with the FDA, Pfizer voluntarily withdrew the new drug application for GO in the U.S. in 2010 as a result of the outcome of S0106, specifically the lack of overall survival benefit in the entire study cohort and the increased rate of early mortality in the GO arm (154). GO was withdrawn from the U.S. and European markets, but continues to be commercially available in Japan, where it has received full regulatory approval (155).

8. BIOMARKERS OF GO'S CLINICAL EFFICACY

The biomarker that has thus far been most recognized as predictor of GO's clinical efficacy with regard to improvement of survival is, as discussed in the previous section in detail, the cytogenetic/molecular profile of the leukemia. In addition, emerging data suggest that CD33 SNPs may prove useful as biomarkers for long-term benefit of GO as well. A number of other AML cell-associated factors – including drug efflux activity, CD33 expression levels, CD33 saturation, circulating CD33 antigenic load, and perhaps methylation status of SOCS3 – have been described as predictors of short-term response,

i.e. reduction in tumor cell loads and/or achievement of CR, but it is unclear whether they could also serve as predictors of long-term benefit. Moreover, it has not been studied to what degree these factors underlie the observed relationship between disease risk and GO efficacy.

Consistent with preclinical studies indicating the central role of drug efflux for GO-induced cytotoxicity, correlative studies on biospecimens from patients enrolled in GO monotherapy trials showed an association between P-glycoprotein as well as MRP1 activity with persistence of marrow blasts, failure to achieve CR, or reduced *in vitro* drug-induced apoptosis (143, 156). This susceptibility to drug efflux may significantly limit the efficacy of GO in clinical practice, especially for the treatment of relapsed or refractory non-APL AML. It is well established that ABC transporter activity, in particular mediated by P-glycoprotein, predicts for therapeutic failure of standard induction therapy (157); thus, the same factor that predicts for failure of conventional chemotherapy and need for salvage therapy also predicts for failure of GO. Additionally, increasing evidence links ABC drug transporters to protection of cancer stem cells – the intended targets for GO – from chemotherapeutic agents (158). In contrast to drug efflux, the effect of CD33 expression on GO efficacy was initially uncertain, and several smaller studies did not find a correlation between CD33 expression levels and response to GO (103, 122). However, later correlative studies conducted on specimens from patients enrolled on the phase 2 trials with GO monotherapy found higher CD33 expression levels on AML blasts to be associated with favorable outcome after GO monotherapy, although multivariate analyses suggested that drug efflux was the more relevant factor for clinical GO resistance than CD33 expression levels (159). A conceivable explanation for this difficulty to demonstrate a quantitative relationship between CD33 expression and clinical efficacy of GO could include the recent observation that CD33 expression levels are inversely associated with favorable cytogenetic/molecular disease features (160) or the possibility that CD33 abundance on disease-relevant stem/progenitor subsets of AML cells may not be adequately reflected by the average CD33 expression of bulk blasts. In line with the former notion, older data, again derived from pediatric AML, indicated that AML patients with high CD33 expression on AML blasts have worse outcomes than those with low CD33 expression when treated with conventional chemotherapy (161). Undoubtedly, this relationship between CD33 expression and inherent AML disease biology could modify the association between CD33 expression and GO efficacy and render such correlative studies challenging. To make matters more complex, recent data raised the possibility that some CD33 SNPs may not only be associated with CD33 expression levels but also response to GO-containing chemotherapy (162). Specifically, homozygosity for the variant allele (TT) at the coding SNP, rs12459419 (C>T; A14V), homozygosity of the reference allele (AA) at the coding SNP, rs2455069 (A>G; R69G), and the 3' UTR SNP, rs1803254 (G>C) have been associated with significantly lower CD33 expression on AML blasts as compared to other genotypes. Moreover, among Caucasians, homozygosity (GG) at the coding SNP, rs35112940 (G>A; R304G), was independently associated with improved relapse-free survival relative to the other genotypes in a cohort of pediatric patients treated with GO-containing multi-agent chemotherapy (AAML03P1) but not in a cohort of pediatric patients treated with multi-agent chemotherapy that did not contain GO (St. Jude AML02 trial) (163). Whether these SNPs directly influence the function of CD33 and response to GO is currently under active investigation.

Considering the relationship between GO uptake and efficacy, it is not surprising that *in vitro* studies have linked reduced CD33 saturation to reduced GO-induced cytotoxicity (103). While the clinically used doses of GO are typically saturating, a high CD33-antigenic load in the peripheral blood probably can act as antibody sink and lead to reduced CD33 saturation in the marrow, thereby adversely affecting GO efficacy (103). An important yet neglected aspect of CD33 uptake is the timing of GO administration. Early studies indicated that surface CD33 levels return to pretreatment levels within 72 hours after anti-CD33 antibody administration despite internalization and modulation (55, 70). This observation suggests that repeated administrations of lower, (near-)saturating doses of GO every 3 days, as clinically pioneered by the ALFA group (143-145), may enhance intracellular accumulation of the calicheamicin-γ_1^I derivative over the initial biweekly administration schedule.

Finally, preliminary data suggest that the methylation status of SOCS3 may serve as biomarker of responsiveness to GO. Specifically, in an uncontrolled, retrospective study on 24 patients treated with GO alone or in combination with chemotherapy at a single institution, methylation of the SOCS3 CpG island was found in 8; there was a statistically insignificant increase in response rate (86% vs. 56%; P=0.17) and longer overall survival (25.1 vs. 10.3 months; P=0.09) in patients with SOCS3 hypermethylation (164).

9. CLINICAL TOXICITIES OF GO

At 9 mg/m^2, GO commonly causes acute infusional reactions, with ~30% and 10% of patients experiencing grade 3/4 infusion-related events after the first and second dose, respectively. In the registration trials, such toxicities included chills (8%), fever (6%), hypotension (4%), nausea (3%), and hypertension (2%). Of note, hypotension – typically transient and fluid responsive – could occur several hours after completion of drug administration (130). Corticosteroids are effective in reducing these reactions (165). Subsequent other common grade 3/4 toxicities include invariable myelosuppression (neutropenia [98%], thrombocytopenia [99%]), transient and reversible liver enzyme abnormalities (hyperbilirubinemia [29%], elevation in asparate aminotransferase [18%] or alanine aminotransferase [9%]), sepsis (17%), fever (13%), chills (9%), nausea/emesis (10%), pneumonia (8%), dyspnea (8%), hypertension (8%), hypotension (8%), asthenia (6%), and neutropenic fever (6%) (130). As a curious rare side effect possibly related to

effective elimination of $CD163^+$ macrophages/monocytes, isolated cases with severe toxic symptoms during intravascular hemolysis secondary to impaired hemoglobin scavenging have been reported in children (166).

A characteristic adverse event that was noticed from the beginning of clinical drug testing is sinusoidal obstruction syndrome (SOS) or veno-occlusive disease (VOD) (129, 130, 167). GO-associated SOS presents as tender hepatomegaly, portal hypertension, fluid retention, weight gain as well as ascites and encephalopathy at later stages, with ascites being pathognomonic for the disease (168). Developing a median of 10-14 days following GO treatment, SOS is more likely when the drug is given at doses higher than 6 mg/m^2 or when it is combined with a hepatotoxic agent (e.g. thioguanin); in the initial clinical studies, SOS was observed most commonly when GO was used pre-transplant, in particular – in up to 65-90% in small patient series – when patients underwent allogeneic HCT within 3-4 months of receiving GO (169, 170). A small study suggested the possibility that the risk of SOS might be modified by a SNP within the glutathione-S-transferase genes, but this link is currently not firmly established (53). The identification of several GO-associated SOS cases shortly after drug approval led the FDA to request the establishment of an industry-sponsored prospective observational registry. Experience from this registry suggested a rate of SOS of around 10% (14.9% and 8.2% in patients undergoing or not undergoing HCT, respectively) (170). SOS proved highly fatal: among 99 cases captured in the FDA's adverse drug event reporting system, 80% required hospitalization and 66% ultimately died (170). Treatment options for GO-associated SOS are poorly defined but limited data indicate that defibrotide may have a role in either prophylaxis or treatment of this condition (120, 171, 172). The etiology of SOS remains somewhat elusive although, based not only on clinical but also histological findings, toxic effects on cells in hepatic sinusoids are likely underlying this pathologic process. Proposed mechanisms include exposure to unconjugated calicheamicin-γ_1^I in the circulation, nonspecific uptake of GO by $CD33^+$ Kupffer cells, or CD33-mediated uptake of GO by one or more of the cell populations in the liver (173); indeed, some data suggest that perhaps, CD33 is also found on hepatocytes (174).

Not surprisingly, data from trials in which GO has been used at lower doses indicate that dose is the key element contributing to GO toxicity. In fact, in most of the recent phase 3 trials, there were no major differences with regard to non-hematologic toxicities between the GO-containing and the control arm when GO was added to conventional induction chemotherapy. Specifically, in the MRC/NCRI AML15 and AML16 trials, hematologic recovery was identical in both arms although patients treated with GO required more platelet transfusions and more days of intravenous antibiotics (146, 148). In contrast, in the ALFA trial, prolonged recovery of neutrophil and platelet counts were observed in the GO arm (145), possibly as a reflection of the more intense, fractionated dosing rather than single administration of GO. SOS was observed very infrequently but remained highly fatal, with 2 out of 3 affected patients dying as a consequence of this toxicity (145).

10. CONCLUSION

Emerging data from large, well-controlled trials indicate that GO benefits many but not all patients with AML, supporting the conclusion that CD33 is a valid target for some disease subsets. Therefore, the current unavailability of GO in many parts of the world is unfortunate, a fact that has led to repeated calls from leading AML experts to both manufacturer and regulatory authorities to reconsider the drug's value and grant selected patients access to this antibody-drug conjugate (58, 175-178).

The long-term benefit of GO in APL and other favorable-risk leukemias, and possibly some leukemias with less favorable prognoses, would be consistent with an ablative effect on $CD33^+$ leukemic stem or progenitor cells in these cases. In view of the available clonal analyses and clinical data with unconjugated anti-CD33 antibodies, this possibility is perhaps most suggested for APL, but high expression of CD33 and low drug efflux activity may partially explain its exquisite sensitivity toward GO. Remarkably, studies have yet to determine whether GO, besides "debulking" of mature $CD33^+$ progeny, can directly kill $CD33^+$ AML stem cells, and whether improved survival from GO is related to successful targeting of such cells (58), a concept that warrants further experimental investigations.

The clinical testing of GO has clearly demonstrated that CD33 is a challenging target for toxin-loaded antibodies: antigen expression at relatively low abundance, slow conjugate internalization, and ABC drug transporter activity limit intracellular toxin accumulation in AML cells and may account at least partly for this difficulty (58). Considering these limitations, rational strategies to improve CD33-targeted immunotherapy would include those that increase CD33 expression/turnover or inhibit drug efflux (179). Additionally, interference with pro-/anti-apoptotic signaling downstream of the toxin-inflicted cellular damage may lower the apoptotic threshold and increase cellular toxicity of GO. An alternative entails the development of novel immunoconjugates employing more potent toxins that are less prone to extrusion by ABC transporters, or anti-CD33 antibody-based therapeutics that utilize fundamentally different mechanisms of actions, e.g. engagement of the host's immune system for effective elimination of AML. While an antibody-drug conjugate that utilized a thiol-containing maytansine derivative recently failed in early clinical testing (180), other CD33-targeting agents have been developed and show promising preclinical activity (181-184).

Last but not least, the experience with GO is an important reminder of the pitfalls of taking a 'one size fits all approach' in drug development for AML, when often benefits are limited to certain groups of patients. As we learn to identify those AMLs likely to respond to certain agents such as CD33-directed antibody-drug conjugates,

we may restrict trial eligibility to patients with these specific leukemias. As a consequence, greater emphasis will be placed on recruiting adequate numbers of patients for the testing of these drug, and our efforts either need to focus more on collaborative, international studies or, more likely, be willing to accept higher false positive and false negative rates or detection of only relatively large differences between treatment and controls (185). With any luck, the lessons learned with GO will help to shift the drug approval process away from dependence on results seen in all AML patients towards the recognition that this disease, like many others, is intrinsically heterogeneous and must be treated accordingly.

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Abbreviations: ALFA, Acute Leukemia French Association; AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; DFS, disease-free survival; ECOG, Eastern Cooperative Oncology Group; ECS, Elongin B/C-Cul2/Cul5-SOCS-box protein; EFS, event-free survival; FDA, U.S. Food and Drug Administration; GO, gemtuzumab ozogamicin; GOELAMS, Groupe Oest-Est d'étude des Leucémies Aiguës et autres Maladies du Sang; HCT, hematopoietic cell transplantation; HOVON, Dutch-Belgian Cooperative Trial Group for Hematology-Oncology; ITIM, immunoreceptor tyrosine-based inhibitory motif; MRC/NCRI, Medical Research Council/National Cancer Research Institute; ORR, overall response rate; OS, overall survival, Siglec, sialic-acid-binding immunoglobulin-like lectin; SH2, Src homology-2; SHP-1/2, SH2 domain containing phosphatase-1/2; SNP, single nucleotide polymorphism; SOCS3, suppressor of cytokine signaling 3; SOS, sinusoidal obstruction syndrome; SWOG, Southwest Oncology Group; U.S., TRM, treatment-related mortality; United States; VOD, veno-occlusive disease

Key Words: AML, Antibody, Calicheamicin, CD33, Gemtuzumab ozogamicin, Immunoconjugate, Review

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