

Multidrug transporter proteins and cellular factors involved in free and mAb linked calicheamicin- γ 1 (gentuzumab ozogamicin, GO) resistance and in the selection of GO resistant variants of the HL60 AML cell line

MAURIZIO CIANFRIGLIA, ALESSANDRA MALLANO, ALESSANDRO ASCIONE and MARIA LUISA DUPUIS

Section of Pharmacogenetics, Drug Resistance and Experimental Therapeutics, Department of Therapeutic Research and Medicines Evaluation, Istituto Superiore di Sanità, Viale Regina Elena 299, Rome, Italy

Received July 31, 2009; Accepted December 23, 2009

DOI: 10.3892/ijo_00000638

Abstract. In this study we elucidated the role of ATP-binding cassette (ABC) multi-drug transporter proteins and cellular factors such as Bcl-2 expression and CD33 down-modulation contributing to free and hP67.6 mAb linked calicheamicin- γ 1 (CalC- γ 1) resistance. We analyzed in a well designed HL60 cell system the relationship between the expression of ABC proteins, Bcl-2 and CD33 modulation with the activity of free and mAb-linked CalC- γ 1. The results herein reported and discussed, strongly suggest that both MDR1-Pgp and MRP1 efflux systems are engaged by CalC- γ 1, but only MDR1-Pgp over-expression efficiently abrogates drug cytotoxicity in MDR cells. Paradoxically, Bcl-2 expression, as observed for other anticancer compounds belonging to the enediyne family of drugs, confers CalC- γ 1 susceptibility rather than resistance in HL60 cells. Further, the isolation of a resistant HL60 subline (HL60AL) that was developed by exposing the parental sensitive cells to sub-effective doses of gentuzumab ozogamicin (GO) over an extended period of time shows a reduced level of CD33 expression that represents an important escape mechanism of HL60 MDR cells to the cytotoxic effect of GO.

Introduction

Gentuzumab ozogamicin (GO) is the first clinically validated cytotoxic immunoconjugate in which a humanised anti-CD33 antibody (hP67.6) is covalently linked to a derivative of

Calicheamicin- γ 1 (CalC- γ 1) (1). More recently, the therapeutic potential of CD22-specific antibody-targeted chemotherapy using inotuzumab ozogamicin (CMC-544) is being evaluated at present in Phase I clinical trials in patients with non-Hodgkin's lymphoma (2). Further, a number of tumor-targeted immunoconjugates of CalC- γ 1 is being explored preclinically for their therapeutic potential (3,4). However, several factors may affect the efficacy of CalC- γ 1 immunoconjugates. These include the common issues of mAb therapy such as antibody specificity, heterogeneous target antigen expression and large inter-individual differences in cellular sensitivity to CalC- γ 1 (5). Further, among individuals with *de novo* AML, the over-expression of the MDR1-P-glycoprotein (MDR1-Pgp), a 170-kDa protein that belongs to the ABC superfamily of proteins, is identified in the 20-75% of malignant cells (6). MDR1-Pgp acts as an efflux pump to remove anticancer drugs from cells, resulting in a simultaneous cross-resistance or multi-drug resistance (MDR) to various chemotherapeutics (7). Although MDR1-Pgp appears to be of biologic and clinical relevance (8), other ABC proteins may be involved in the outcome of GO-treated AML patients (9,10). One such protein, the multi-drug resistance-associated protein (MRP1), is distantly related to MDR1-Pgp, and like MDR1-Pgp, lowers intracellular drug accumulation by promoting drug efflux and MDR (11).

Previous studies hypothesized an association between MDR1-Pgp and MRP1 expression and clinical responses to GO (8,12,13), others reported discrepant results attributed to the multi-factorial nature of drug resistance (14,15). Engagement of CD33 by GO results in immunoconjugate internalization and hydrolytic release of the toxic CalC- γ 1 moiety which causes DNA damage and cell death (1). Even though, CD33 expression and related pathways involved in GO-induced cytotoxicity are the object of several studies, the resistance mechanism emerging from CD33-GO interaction is not yet fully understood (15).

In order to elucidate the cellular factors contributing to free and mAb linked CalC- γ 1 resistance a well designed HL60 MDR cell system was used in parallel with drug-sensitive HL60 cells to analyze: i) the ability of CalC- γ 1 to interact with the MDR1-Pgp and MRP1 drug efflux systems expressed in MDR variants, ii) the role of MDR1-Pgp and

Correspondence to: Dr Maurizio Cianfriglia, Section of Pharmacogenetics, Drug Resistance and Experimental Therapeutics, Department of Therapeutic Research and Medicines Evaluation, Istituto Superiore di Sanità, Viale Regina Elena 299, Rome, Italy
E-mail: maurizio.cianfriglia@iss.it

Key words: multi-drug resistance, calicheamicin, tumor-targeting, immunoconjugates, gentuzumab ozogamicin

MRP1 in conferring free and mAb linked CalC- γ 1 resistance and nature of cell death in CalC- γ 1 induced cytotoxicity, and iii) the effect of GO treatment on CD33 expression and its role in GO resistance. The results herein reported and discussed, strongly suggest that both MDR1-Pgp and MRP1 efflux systems are engaged by CalC- γ 1, but only MRP1-Pgp over-expression efficiently abrogated drug cytotoxicity in MDR cells. In addition, CalC- γ 1 exerts potent cytotoxicity via necrosis or apoptosis; the latter cell death mechanism seems to be paradoxically enhanced by the expression of the anti-apoptotic Bcl-2 expression. Moreover, at least in our experimental conditions, the CD33 down-modulation represents an important escape mechanism of HL60 cells from the cytotoxic effect of GO.

Materials and methods

Cell lines. The parental drug-sensitive HL60 cell line and its multi-drug resistant (MDR) variants HL60/DNR and HL60/ADR were kindly provided by Dr R. Tang (Paris) (16). Cells were grown under standard conditions for mammalian cells cultured in suspension. The basic medium (BM) for cell culturing consisted of RPMI-1640 supplemented with 10% fetal calf serum (FCS), L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 U/ml). All these components were purchased from Hyclone (Logan, UT). Identical BM and culture conditions were used to select GO resistant variant and the relationship between Bcl-2 expression and CalC- γ 1 activity in HL60 cells (HL60T, HL60C) obtained from ATCC (Manassas, VA) and separately cultured in two laboratories (Testa, ISS, Rome; Cianfriglia, ISS, Rome, Italy).

mAbs and cell phenotyping. The phenotype of the parental drug-sensitive HL60 cell lines and its MDR variants HL60/DNR and HL60/ADR was defined by incubating samples of intact/living cells (1×10^6 /ml) with 5 μ g/ml of the MDR1-Pgp-specific mAb MM4.17 (17) or CD33 specific mAb hP67.6 (Wyeth Pharmaceuticals, Collegeville, PA). MRP1 expression was assessed with the MRP1-specific mAb MRPm6 (Vinci-Biochem, Firenze, Italy) in fixed/permeabilized cells as previously described (18). After 1 h, cells were washed and incubated again with fluorescein isothiocyanate-labeled secondary mouse antibody (FITC-GAM, Cappel, West Chester, PA, USA) and subjected to FACScalibur flow cytometer analysis (Becton-Dickinson, San Jos , CA).

MDR efflux assay. HL60, HL60/DNR and HL60/ADR cells (1×10^6) were loaded with doxorubicin (5 μ g/ml) in 1 ml of BM in the presence of a several CalC- γ 1 concentrations ranging from 10 μ g/ml to 5 ng/ml and verapamil (MDR1-Pgp inhibitor, 2.5 μ g/ml, Isoptin, Abbott, Campoverde di Aprilia, LT, Italy) or MK-571 (MRP1 inhibitor; 25 μ M, Vinci-Biochem) for 1 h at 37°C. The cells were incubated with doxorubicin only or drug diluent in parallel cultures. At the end of incubation, the cells were washed in serum-free medium and resuspended in BM in the presence of CalC- γ 1 (Wyeth), verapamil (Abbott) or MK-571 (Vinci-Biochem) or drug diluent in control samples for a further 1 h at 37°C. Finally, cells were washed twice with ice-cold PBS/FACS, and analyzed in a flow cytometer (Becton-Dickinson).

Cytotoxicity assay. The evaluation of cell viability at different CalC- γ 1 or GO concentrations was determined by PreMix WST-1 kit (Vinci-Biochem) according to the manufacturer's instructions. Briefly, HL60, HL60/DNR and HL60/ADR cells in exponential phase of growth were collected, extensively washed with warm RPMI-1640 and resuspended at the concentration of 1×10^5 cells/ml in BM. Cells were seeded (in triplicate) in 96-wells Costar plates (Costar, Rochester, NY) in presence of different CalC- γ 1 or GO concentrations and incubated for 48 h at 37°C in 5% CO₂. Within its inhibitory range, the drug decreased growth of all cell lines proportionally to drug concentration. After the incubation period, 20 μ l/well of PreMix WST-1 reagent was added and the cells were incubated for 1 h in the above reported conditions. Finally, the absorbance of the samples was measured using a microtiter plate ELISA reader at 450 nm. The relative cell growth was calculated as percentage of the untreated control culture. The obtained dose-response profile fulfilled the concentration inhibiting growth by 50% (EC₅₀).

Cell cycle and apoptosis. For cytofluorimetric evaluation of apoptosis, HL60, HL60DNR and HL60ADR cells were adjusted in BM at the final concentration of 4×10^5 cells/ml and plated in 24-well Costar plates, in the presence of the CalC- γ 1 (from 1 to 50 ng/ml). After 24 h of growth the cells were harvested and washed twice in PBS, resuspended in binding buffer (2×10^5 cells/ml) and stained by annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Vinci-Biochem) for 10 min at room temperature (RT). After washing, the cells were stained with propidium iodide (PI) stock solution, and immediately analyzed on a FACScalibur flow cytometer (BD) equipped with a 488 nm argon laser. For cell cycle analysis, 4×10^5 cells were resuspended in PBS and were diluted 1:1 with the staining solution 0.1% Triton X-100 (Sigma), 0.04% EDTA (Sigma), 50 μ g/ml PI (Calbiochem) in PBS and adding 10 μ g di RNase (Sigma). The cell suspension was incubated for 30 min at RT, after the cells were analyzed by FACScalibur instrument.

Bcl-2 expression. For Bcl-2 determination, cells were washed twice in PBS, lysed at 4°C for 30 min in ice-cold buffer (pH 7.5) constituted by 50 mmol/l Tris-HCl, 150 mmol/l NaCl, 1% nonidet P-40, 20 μ g/ml aprotinin, 2 mmol/l phenylmethyl-sulphonyl fluoride and 10 mg/ml leupeptin. Cellular debris were removed by centrifugation at 12000 g for 20 min at 4°C, and the supernatants were frozen until required. Fifty micrograms of proteins were separated on sodium dodecyl sulphate (SDS) 12% polyacrylamide gels and electroblotted on nitrocellulose membranes (Bio-Rad, Hercules, CA) that were blocked in PBS with 0.05% Tween-20 containing 5% non-fat dry milk. Anti-Bcl-2 mAb (R&D Systems, Minneapolis, MN) was used at 0.1 μ g/ml, 4°C overnight (ON) and anti- β -actin (Sigma-Aldrich, St. Louis, MO) was used at 0.2 μ g/ml, RT 1 h. Specific binding was detected by incubation with an horseradish peroxidase-conjugated anti-mouse IgG at 0.2 μ g/ml at RT 1 h (Dako, Glostrup, Denmark) by enhanced chemiluminescence kit (Pierce, Rockford, IL).

Selection of GO resistant HL60 cells. HL60C cell line were cultured for 8 weeks in the presence of 5 ng/ml of GO in BM

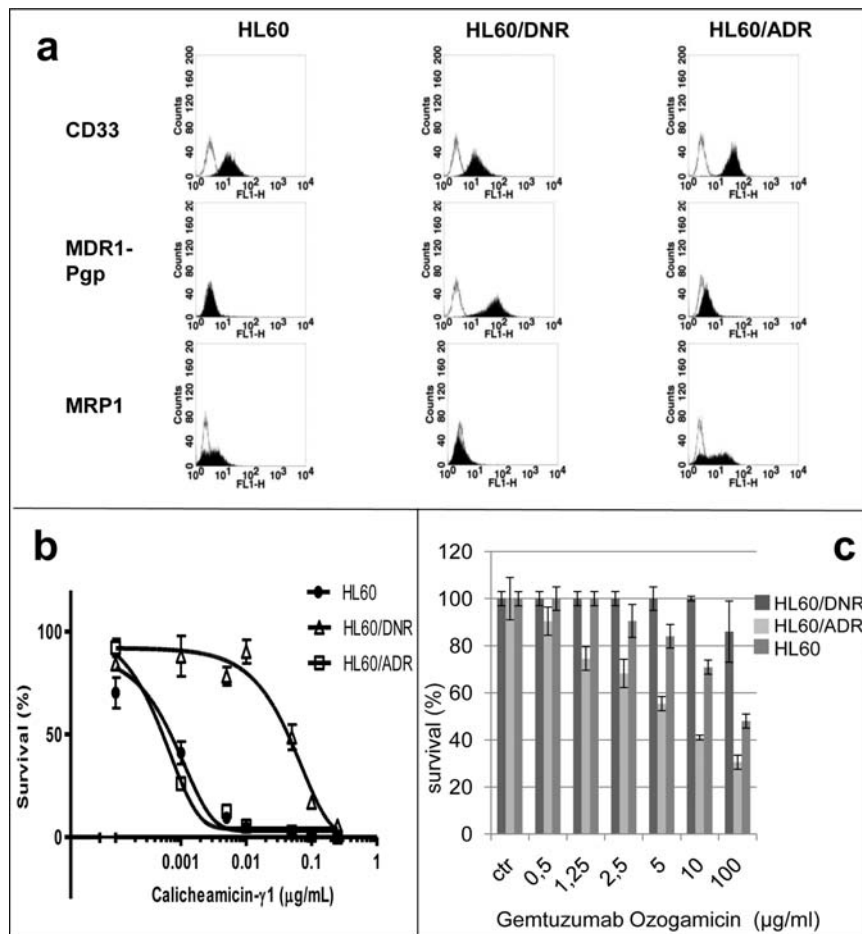


Figure 1. Expression of ABC transporters and CalC- γ 1 cytotoxicity. Living/intact HL60 cells were stained with CD33 specific mAb hP67.6 and MDR1-Pgp-specific mAb MM4.17. Fixed/permeabilized HL60 cells were stained with MRP1-specific mAb MRPm6 (a). Cytotoxicity of free or antibody-conjugated CalC- γ 1 (GO) was determined by WST-1 assay after 48-h treatment with CalC- γ 1 (b) or GO (c) at the indicated concentrations. Results are the mean of three independent experiments, each performed in triplicate.

containing medium. As control, in parallel culture HL60C cells were treated with 5 ng/ml of the anti-CD33 mAb hP67.6. Revertants of the HL60C GO resistant cell line were obtained by culturing the cells for 2 weeks in GO free-BM.

Data presentation. All the experiments were repeated at least in triplicate. The significance was assessed by Student's t-test. The criterion for statistical significance was set at $P < 0.05$.

Results and Discussion

MDR phenotype of HL60 cells. Both MDR1-Pgp and MRP1 expression are involved in drug transport and may determine, in adults suffering from AML treated with GO the amount of CalC- γ 1 that is available in the leukemic cells to induce apoptosis (15). The tight relationship between expression of one or both of these ABC transporters and adverse outcome of GO monotherapy is proposed on the basis of clinical investigation and/or *ex vivo* analysis of AML blast samples. In contrast, very few studies have been conducted to verify as to whether MDR1-Pgp and MRP1 expression confer CalC- γ 1 resistance in *in vitro* conventional approach consisting of the comparison of sensitive cells with counterparts that were experimentally induced to become MDR (19).

In transformed cells and under selective pressure, the MDR phenotype both *in vivo* and *in vitro* systems is the consequence of very complex biological phenomena which include genetic regulation of expression of different ABC transporters. For example, it is hypothesized in AML cells that the over-expression of MRP1 gene preceded that of the MDR1 gene and afterward MRP1 and MDR1 may be co-over-expressed (20-22). Finally, by a further increase of the selective pressure condition, the MDR1-Pgp may emerge as the unique and very efficient drug transporter machinery expressed on MDR cells. A similar scenario of expression of ABC proteins is found in HL60 MDR cells used in this study (Fig. 1a). By using mAbs MM4.17 and MRP1m6 specifically recognizing external and cytoplasmic domains of MDR1-Pgp and MRP1 respectively, we found that the parental drug-sensitive HL60 cells are completely negative for MDR1-Pgp while a small fraction of cells (from 10 to 15%) are found MRP1⁺. Afterwards, MRP1 over-expression is observed after a first step of selection coincident with the isolation of HL60/ADR cells. A subpopulation of these MDR variants also reacted with the mAb MM4.17 confirming that MDR1-Pgp and MRP1 may be co-over-expressed. Finally, HL60/DNR MDR cells are characterized by a very high level of MDR1-Pgp and abrogation of MRP1 expression. These findings indicate that the HL60 cells we used are an

appropriate *in vitro* system to study the correlation between MDR phenotype and free or mAb linked CalC- γ 1.

Cytotoxicity of CalC- γ 1 on HL60 cells. CalC- γ 1 belongs to enediyne family of antibiotics and these molecules (23,24) are similar in size and structure to known substrates of the MDR1-Pgp. Early studies conducted in drug-sensitive/resistant cell pairs with mAb-linked CalC- γ 1 indicated a modulating effect of MDR1-Pgp on cytotoxicity (19). In addition it is reported that in AML blast cells the MDR1-Pgp expression correlated with treatment failure among patients receiving GO and that MRP1 can also attenuate GO-induced cytotoxicity (6). GO acts as cytotoxic agent via a cascade of events which include mAb binding, internalization, translocation into lysosomes and finally, an enzymatic pH-dependent release of CalC- γ 1 derivative that ultimately leads to site-specific and/or conformation-dependent single- and double-stranded DNA scission (1,15). Hence, a plethora of cellular factors are involved in CalC- γ 1 mAb mediated cytotoxicity. In order to elucidate the role of MDR1-Pgp and MRP1 in conferring CalC- γ 1 resistance, the parental HL60 cell line and its MDR variants were tested for free and mAb conjugated-CalC- γ 1 activity using cell growth experiments. The drug-response profiles obtained by WST-1 assay (Fig. 1b) revealed after 48 h that free CalC- γ 1 exerts a very potent cytotoxic activity on the parental HL60 cells ($EC_{50} = 0.78 \pm 0.15$ ng/ml) while HL-60/DNR ($EC_{50} = 46 \pm 7.3$ ng/ml) behaves as 'classical' MDR cell variant being about 59-fold more resistant than parental cells ($P < 0.05$). Surprisingly, the HL60/ADR cells show a higher sensitivity to the drug ($EC_{50} = 0.47 \pm 0.12$ ng/ml) being more susceptible to CalC- γ 1 than the parental cell line HL60 (Fig. 1b) ($P < 0.05$).

Similar patterns of drug response profiles are observed by treating the HL60 MDR cells with GO thus confirming that free or mAb linked CalC- γ 1 interact with MDR1-Pgp and/or MRP1 once the immunoconjugate is internalized and the linker joining anti-CD33 mAb and CalC- γ 1 derivative is cleaved (Fig. 1c).

MDR1-P-gp and MRP1 drug efflux are differently affected by CalC- γ 1. Doxorubicin is a substrate for several ABC transporters and incubation of cell lines expressing functional MDR1-Pgp or MRP1 with this fluorescent compound, followed by washing and further incubation at 37°C, results in a diminished fluorescence due to active drug efflux (6). In order to clarify if CalC- γ 1 is an MDR1-Pgp or MRP1 substrate we first controlled the functional activity of these drug transporters. As shown in Fig. 2, the MDR1-Pgp and MRP1 inhibitors verapamil and MK-571, block doxorubicin efflux in HL-60/DNR and HL-60/ADR, respectively although they did not affect the intracellular concentration of doxorubicin in the sensitive parental cell line HL60. Conversely, CalC- γ 1 is capable of causing an intracellular accumulation of doxorubicin in a dose-dependent manner in both HL60/DNR and HL60/ADR MDR cells (Fig. 2) with an efficacy that at lower concentrations, greatly exceeds that of MDR modulators (data not shown). However, further and well designed studies are needed to verify if the inhibition of MDR1-Pgp and/or MRP1 by CalC- γ 1 is merely due to competition of doxorubicin and CalC- γ 1 for the same drug binding site or

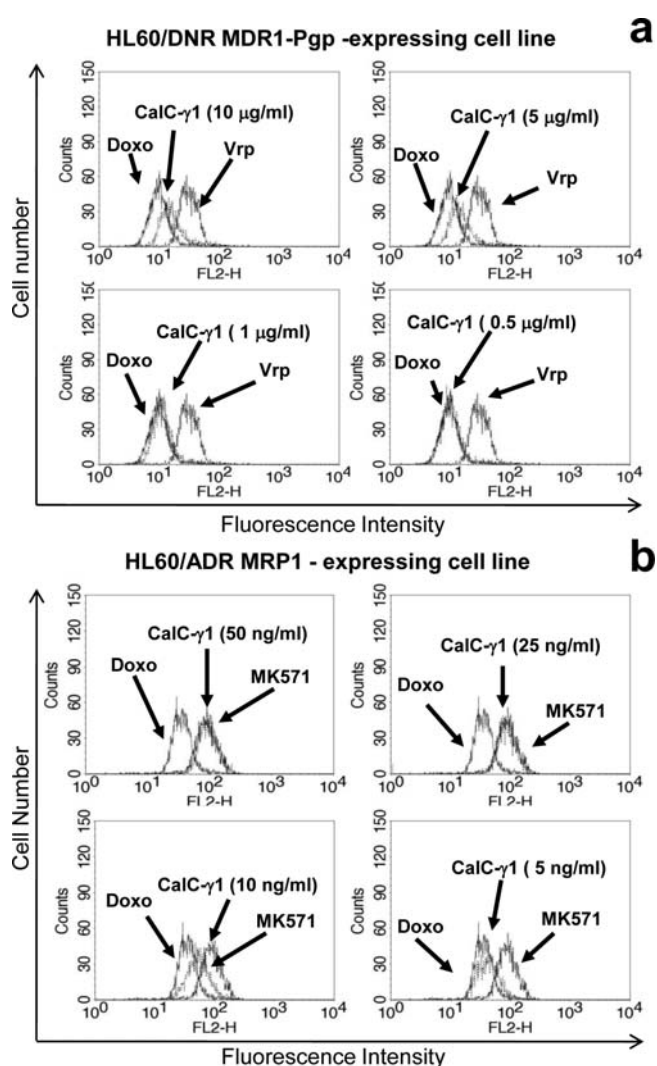


Figure 2. Drug efflux of HL60 cells. HL60/DNR cells were incubated with doxorubicin (5 μ g/ml) and the efflux inhibited by verapamil (Vrp, 2.5 μ g/ml) or CalC- γ 1 at the indicated concentrations (a). Identical procedure was utilized in HL60/ADR cells where the doxorubicin efflux was controlled by 25 μ M of the MRP1 inhibitor MK571 (b).

other mechanisms that similar to the third generation of MDR modulators (25), specifically and potentially inhibit ABC transporters by altering their structure and function.

CalC- γ 1-dependent apoptosis and cell cycle in HL60 cells.

To characterize the type of cell death occurring under CalC- γ 1 treatment, we studied a typical event of apoptosis, namely the presence of phosphatidylserine at the outer face of the plasma membrane in cells cultured in the presence of different concentration of CalC- γ 1. Phosphatidylserine exposure was indeed revealed, after 24-h incubation with the drug, by the binding of annexin-V/FITC. A clear dose-dependent effect of apoptosis was observed in HL60/ADR while the MDR1-Pgp⁺ HL60/DNR cell line are quite insensitive to the treatment of CalC- γ 1 ≤ 50 ng/ml of (Fig. 3a). A further increase of drug concentration caused cell death by apoptosis in these cells but the percent of apoptosis was negligible compared to that observed in HL60/ADR cells. Time course analysis of cell death showed that apoptosis induced by 5 ng/ml of CalC- γ 1 occurred within 8 h only in a small fraction of MRP1⁺

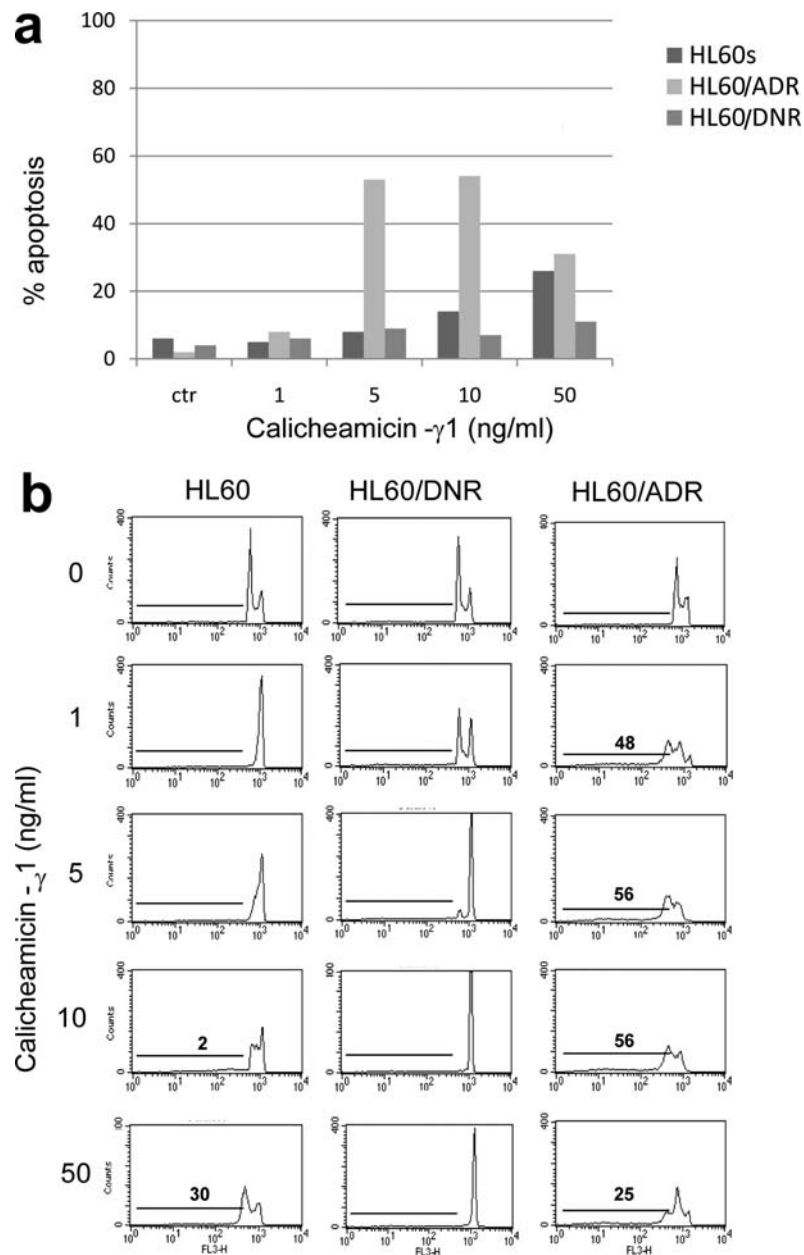


Figure 3. Apoptosis and cell cycle analysis. (a) Apoptosis was monitored after 24-h incubation with different concentrations of CalC- γ 1 using annexin-V/FITC. Cell death was detected by fluorescence-activated cell sorter, using annexin-V/FITC intensity measurements. (b) Flow cytometric analysis of cell cycle was performed on HL60, HL60/DNR and HL60/ADR after 24-h treatment. Cell distribution, according to their DNA content was measured after cell permeabilization and PI staining. The fraction of cells with sub-G1 DNA content (apoptotic cells) was calculated using WinMDI version 2.8 software. The results of a representative experiment are shown.

HL60/ADR cells and continued to increase throughout the incubation period (16 h) reaching the peak at 24 h (data not shown).

To additionally characterize the type of cell death occurring under these conditions, we did flow cytometric analysis of HL60 cell cycle after 24 h of CalC- γ 1 treatment by staining cells with PI. HL60/ADR cell line treated with different amounts of CalC- γ 1 showed a dose-dependent increase of cell population with hypodiploid (sub-G1) nuclei, in which DNA cleavage occurred as the ultimate step of apoptosis. This pathway is absent in H60/DNR cells, in which no increase in sub-G1 peak is observed. Conversely, the pattern of PI staining of HL60 parental cell line is quite similar to that of

HL60/DNR or HL60/ADR cells depending on concentrations of CalC- γ 1 treatment (Fig. 3b).

Bcl-2 content and CalC- γ 1 cytotoxicity. In an attempt to explain the pattern of cell death induced by CalC- γ 1 in HL60/ADR cell line, the expression of Bcl-2 that antagonizes apoptosis in tumor cells was considered. It is well known that tumors, and in particular drug resistant tumor cell lines, may express high levels of this anti-apoptotic protein (26). Western blot analysis shows (Fig. 4) a high expression level of Bcl-2 in HL60/ADR cells while the parental drug-sensitive HL60 cells and its MDR variant HL60/DNR over-expressing MDR1-Pgp have an almost undetectable level of the Bcl-2

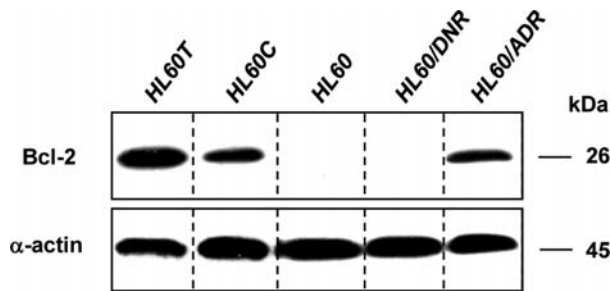


Figure 4. Bcl-2 content by Western blot analysis. Fifty micrograms of total protein extracts from the HL60 cell lines were used for detection of Bcl-2 protein by staining with specific mAbs as described in Materials and methods. β -actin was used as loading control.

protein. The pattern of Bcl-2 expression we found in HL60 MDR cell panel has already described (16) and depicted a paradoxical phenomenon of cytotoxic potentiation rather than resistance conferred by this anti-apoptotic protein. To corroborate the hypothesis that Bcl-2 expression inversely correlated with CalC- γ 1 susceptibility, we examined HL60 cells from different sources (HL60C and HL60T; for details see Materials and methods) that differently from the HL60 cell line we used in the first part of the study, are characterized by high level of Bcl-2. Surprisingly, in these cells, the expression of this anti-apoptotic protein parallels with a higher susceptibility to free CalC- γ 1 (Fig. 5a) and mAb-linked CalC- γ 1 (Fig. 5b).

A paradoxical effect of Bcl-2 has been described (27) in the cytotoxic trial of PC12 pheochromocytoma cells with neocarzinostatin (NCS), a DNA cleaving agents of the enediyne family of drugs. In this study Mi *et al.* (27) demonstrated that the cleavage of Bcl-2 to its proapoptotic counterpart mediated by caspase-3 is responsible for potentiation of apoptosis. More recently, it was reported that neuroblastoma and brain tumor cell lines are highly susceptible to the cytotoxic activity of enediynes. In this article Rogers *et al.* (28) demonstrated that the sensitivity of brain tumor cells to NCS is enhanced by transfection with an expression construct for Bcl-2 and that NCS cytotoxicity it is proportional to the product of the relative contents of Bcl-2 and caspase-3. These data seem in contrast with previous findings that hypothesized a protective effect of Bcl-2 to AML chemotherapy (29). However, we found this paradoxical phenomenon of cytotoxic potentiation in parallel with Bcl-2 expression only in presence of free CalC- γ 1. Nonetheless, the role of Bcl-2 expression in free or mAb linked CalC- γ 1 cell killing is poor investigated and further and well designed studies are required to define the biological function of this anti-apoptotic protein during GO therapy.

CD33 down-modulation and GO resistance. The existence of a quantitative relationship between CD33 expression and *in vitro* response to GO is hypothesized by use of lentivirus-mediated gene transfer to manipulate CD33 expression in myeloid cell lines that normally lack or have very low levels of CD33 (30). Furthermore, AML blasts of patients responsive to the drug were found to have a significantly higher mean CD33 level and lower MDR1-Pgp activity than the non-

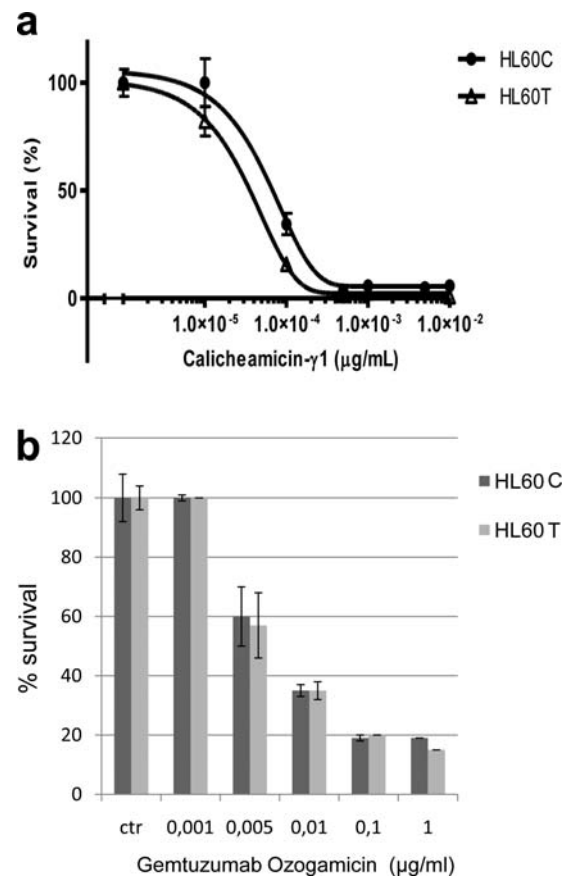


Figure 5. CalC- γ 1 cytotoxicity on HL60 expressing high amounts of Bcl-2. Cell survival was determined by WST-1 assay after 48-h treatment of CalC- γ 1 (a) or GO (b) at the indicated concentrations. LC_{50} was 58 ± 6.9 pg for HL60C and 35 ± 1.3 pg for HL60T cell lines. Results are the mean of three independent experiments, each done in triplicate; bars, \pm SD.

responders, with CD33 expression and MDR1-Pgp activity exhibiting an inverse correlation (12). In contrast, current experience indicates, however, that treatment failure or drug resistance to GO is not commonly associated with outgrowth or selection of CD33-negative leukaemia (15). In addition, there are even doubts of the effective role of CD33 mediating GO cytotoxicity (31,32). In spite of these important questions, little efforts have been applied to study the mechanism of GO acquired resistance in a classical *in vitro* approach, namely the exposure of cells to sub-effective dose of GO and verifying after an extended period of time as to whether, phenotypic alteration of the CD33 may be related with an escape of antibody-mediated cytotoxicity. Our investigation was conducted using the HL60C AML cell line which is characterized by Bcl-2 expression and a CalC- γ 1 susceptibility similar to that observed in the majority of AML blasts.

Then, we exposed HL60C cells to GO at dose of 5 ng/ml that killed 80-90% of the cells in the first round of treatment and then maintained the surviving 10-20% of the cells in an uninterrupted subculture with 5 ng/ml GO for 8 weeks. As control, in parallel culture, HL60C cells were treated with identical modality but in presence of only the anti-CD33 mAb hP67.6. Finally, a GO-resistant cell line named HL60AL was created and investigated for CD33 expression and susceptibility to free- or mAb hP67.6 linked CalC- γ 1.

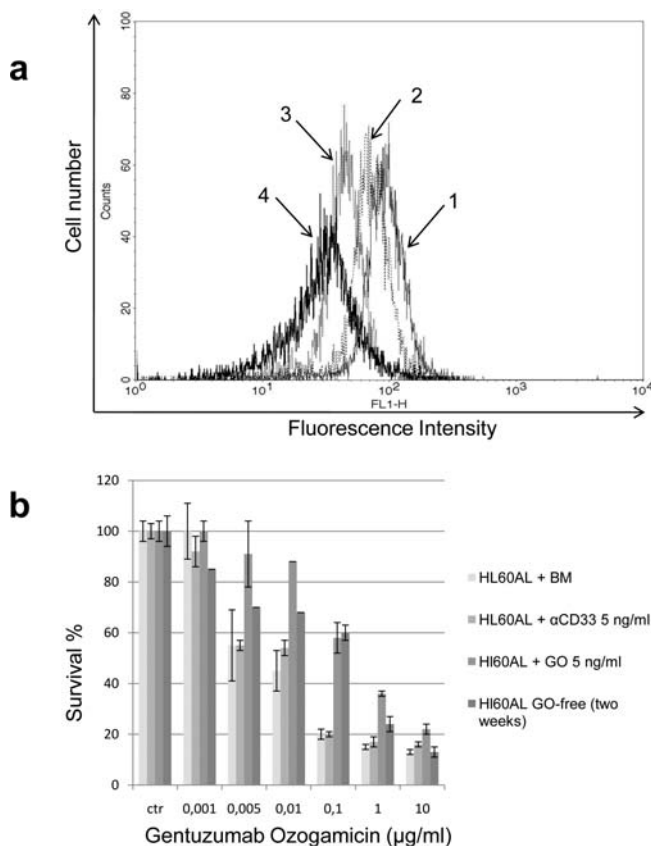


Figure 6. CD33 down-modulation and GO cytotoxicity. (a) Staining of anti-CD33 mAb hP67.6 on parental HL60 cells (1), after GO selection (4) and in parallel culture with only the mAb hP67.6 (2). Staining was also conducted on GO resistant HL60 cell line (revertant) (3) cultured in the absence of the selective agent. Cytotoxicity (b) of antibody-conjugated CalC- γ 1 (GO) was determined by WST-1 assay after 48-h treatment with GO at the indicated concentrations. Results are the mean of three independent experiments, each done in triplicate.

The results reported in Fig. 6, show that the HL60AL cell line has a significant reduction of CD33 expression (Fig. 6a), and in comparison with the parental HL60C cells untreated or treated with only mAb hP67.6 appears more resistant to GO (Fig. 6b). The tight linkage between CD33 expression and GO susceptibility is also demonstrated in revertant cells (Fig. 6) isolated by culturing (for two weeks) HL60AL cells in GO free-medium. These cells regain CD33 expression and GO susceptibility. In addition, the selective pressure utilized for the isolation of GO resistant variants did not induce detectable MDR1-Pgp and MRP1 expression or drug efflux function (data not shown). These findings indicate that, at least in our experimental condition, the CD33 down-modulation may represent an efficient escape mechanism for AML treated with (sub-effective dose) of GO. Nonetheless, important questions raised about the role of CD33 in GO mediated cytotoxicity remain unresolved and require further investigations in a well designed *in vitro* and *in vivo* animal models.

In conclusion, in this study we demonstrated that: i) both MDR1-Pgp and MRP1 efflux systems are engaged by CalC- γ 1 but only MRP1-Pgp over-expression efficiently abrogated drug cytotoxicity and confers an MDR phenotype to HL60 cells, ii) free or mAb hP67.6-linked (GO) CalC- γ 1 exerted, in parental HL60 cell line and its HL60/ADR MDR variant over-expressing MRP1, potent cytotoxicity, and iii) the anti-

apoptotic protein Bcl-2 displayed a paradoxical phenomenon of potentiation rather than resistance to the cytotoxic activity of free CalC- γ 1. Moreover, we found that CD33 down-modulation represents an efficient drug resistance mechanism as response to GO treatment. In conclusion, MDR1-Pgp and CD33 targeting antigen expression, in our experimental conditions, are two biological factors playing a key role in the efficacy of CalC- γ 1-immunoconjugation.

Acknowledgements

This work was supported in part by funds from the ISS-NIH and from ISS-Italian Ministry of Health research projects in Oncology. A.M. is recipient of a fellowship granted by ACC-ISS research project. We thank Wyeth Pharmaceuticals, Collegeville, PA, USA and Wyeth, Aprilia, LT, Italy for kindly providing gentuzumab ozogamicin, calicheamicin- γ 1 and the anti-CD33 mAb hP76. We thank Maura Cianfriglia for editing this manuscript.

References

1. Stasi R, Evangelista ML, Buccisano F, Venditti A and Amadori S: Gemtuzumab ozogamicin in the treatment of acute myeloid leukemia. *Cancer Treat Rev* 34: 49-60, 2008.
2. Di Joseph JF, Dougher MM, Kalyandrug LB, Armellino DC, Boghaert ER, Hamann PR, Moran JK and Damle NK: Antitumor efficacy of a combination of CMC-544 (inotuzumab ozogamicin), a CD22-targeted cytotoxic immunoconjugate of calicheamicin, and rituximab against non-Hodgkin's B-cell lymphoma. *Clin Cancer Res* 12: 242-249, 2006.
3. Di Joseph JF, Dougher MM, Armellino DC, Kalyandrug L, Kunz A, Boghaert ER, Hamann PR and Damle NK: CD20-specific antibody-targeted chemotherapy of non-Hodgkin's B-cell lymphoma using calicheamicin-conjugated rituximab. *Cancer Immunol Immunother* 56: 1107-1117, 2007.
4. Damle NK: Tumour-targeted chemotherapy with immunoconjugates of calicheamicin. *Expert Opin Biol Ther* 4: 1445-1452, 2004.
5. Goemans BF, Zwaan CM, Vijverberg SJ, Loonen AH, Creutzig U, Hählen K, Reinhardt D, Gibson BE, Cloos J and Kaspers GJ: Large interindividual differences in cellular sensitivity to calicheamicin may influence gemtuzumab ozogamicin response in acute myeloid leukemia. *Leukemia* 22: 2284-2285, 2008.
6. Leith CP, Kopecky KJ, Chen IM, Eijdens L, Slovak ML, McConnell TS, Head DR, Weick J, Grever MR, Appelbaum FR and Willman CL: Frequency and clinical significance of the expression of the multidrug resistance proteins MDR1/P-glycoprotein, MRP1, and LRP in acute myeloid leukemia: a Southwest Oncology Group Study. *Blood* 94: 1086-1099, 1999.
7. Gottesman MM, Fojo T and Bates SE: Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat Rev Cancer* 2: 48-58, 2002.
8. Linenberger ML, Hong T, Flowers D, Sievers EL, Gooley TA, Bennett JM, Berger MS, Leopold LH, Appelbaum FR and Bernstein ID: Multidrug-resistance phenotype and clinical responses to gemtuzumab ozogamicin. *Blood* 98: 988-994, 2001.
9. Bendorra Z, Faussat AM, Sayada L, Perrot JY, Tang R, Chaoui D, Morjani H, Marzac C, Marie JP and Legrand O: MRP3, BCRP, and P-glycoprotein activities are prognostic factors in adult acute myeloid leukemia. *Clin Cancer Res* 11: 7764-7772, 2005.
10. Ho MM, Hogge DE and Ling V: MDR1 and BCRP1 expression in leukemic progenitors correlates with chemotherapy response in acute myeloid leukemia. *Exp Hematol* 36: 433-442, 2008.
11. Deeley RG and Cole SP: Substrate recognition and transport by multidrug resistance protein 1 (ABCC1). *FEBS Lett* 580: 1103-1111, 2006.
12. Walter RB, Gooley TA, van der Velden VH, Loken MR, van Dongen JJ, Flowers DA, Bernstein ID and Appelbaum FR: CD33 expression and P-glycoprotein-mediated drug efflux inversely correlate and predict clinical outcome in patients with acute myeloid leukemia treated with gemtuzumab ozogamicin monotherapy. *Blood* 109: 4168-4170, 2007.

13. Walter RB, Raden BW, Hong TC, Flowers DA, Bernstein ID and Linenberger ML: Multidrug resistance protein attenuates gemtuzumab ozogamicin-induced cytotoxicity in acute myeloid leukemia cells. *Blood* 102: 1466-1473, 2003.
14. Legrand O, Zompi S, Perrot JY, Faussat AM, Benderra Z, Chaoui D and Marie JP: P-glycoprotein and multidrug resistance associated protein-1 activity in 132 acute myeloid leukemias according to FAB subtypes and cytogenetics risk groups. *Haematologica* 89: 34-41, 2004.
15. Linenberger ML: CD33-directed therapy with gemtuzumab ozogamicin in acute myeloid leukemia: progress in understanding cytotoxicity and potential mechanisms of drug resistance. *Leukemia* 19: 176-182, 2005.
16. Tang R, Faussat AM, Majdak P, Perrot JY, Chaoui D, Legrand O and Marie JP: Valproic acid inhibits proliferation and induces apoptosis in acute myeloid leukemia cells expressing P-gp and MRP1. *Leukemia* 18: 1246-1251, 2004.
17. Cianfriglia M, Willingham MC, Tombesi M, Scagliotti GV, Frasca G and Chersi A: P-glycoprotein epitope mapping. I. Identification of a linear human-specific epitope in the fourth loop of the P-glycoprotein extracellular domain by MM4.17 murine monoclonal antibody to human multidrugresistant cells. *Int J Cancer* 56: 153-160, 1994.
18. Flens MJ, Izquierdo MA, Scheffer GL, Fritz JM, Meijer CJ, Scheper RJ and Zaman GJ: Immunochemical Detection of the multidrug resistance-associated protein MRP in human multidrug-resistant tumor cells by monoclonal antibodies. *Cancer Res* 54: 4557-4563, 1994.
19. Naito K, Takeshita A, Shigeno K, Nakamura S, Fujisawa S, Shinjo K, Yoshida H, Ohnishi K, Mori M, Terakawa S and Ohno R: Calicheamicin-conjugated humanized anti-CD33 monoclonal antibody (gemtuzumab zogamicin, CMA-676) shows cytotoxic effect on CD33-positive leukemia cell lines, but is inactive on P-glycoprotein-expressing sublines. *Leukemia* 14: 1436-1443, 2000.
20. Slapak CA, Mizunuma N and Kufe DW: Expression of the multidrug resistance associated protein and P-glycoprotein in doxorubicin selected human myeloid leukemia cells. *Blood* 84: 3113-3121, 1994.
21. Brock I, Hipfner DR, Nielsen BS, Jensen PB, Deeley RG, Cole SPC and Sehested M: Sequential coexpression of the multidrug resistance gene MRP and mdr1 and their products in VP-16 (etoposide)-selected H69 small cell lung cancer cells. *Cancer Res* 55: 459-462, 1995.
22. Zhou DC, Ramond S, Viguié F, Faussat AM, Zittoun R and Marie JP: Progressive resistance to homoharringtonine in human myeloid leukemia K562 cells: relationship to sequential emergence of MRP and MDR1 gene overexpression and MDR1 gene translation. *Int J Cancer* 65: 365-371, 1996.
23. Nicolaou KC, Dai WM, Tsay SC, Estevez VA and Wrasidlo W: Designed enediynes: a new class of DNA-cleaving molecules with potent and selective anticancer activity. *Science* 256: 1172-1178, 1992.
24. Nicolaou KC, Smith AL and Yue EW: Chemistry and biology of natural and designed enediynes. *Proc Natl Acad Sci USA* 90: 5881-5888, 1993.
25. Wu CP, Calcagno AM and Ambudkar SV: Reversal of ABC drug transporter-mediated multidrug resistance in cancer cells: evaluation of current strategies. *Curr Mol Pharmacol* 1: 93-105, 2008.
26. Reed JC: Bcl-2 family proteins and hematologic malignancies: history and future prospects. *Blood* 111: 3322-3330, 2008.
27. Mi Z, Hong B, Mirnics ZK, Tyurina YY, Kagan VE, Liang Y and Schor NF: Bcl-2-mediated potentiation of neocarzinostatin-induced apoptosis: requirement for caspase-3, sulfhydryl groups, and cleavable Bcl-2. *Cancer Chemother Pharmacol* 57: 357-367, 2006.
28. Rogers D, Nylander KD, Mi Z, Hu T and Schor NF: Molecular predictors of human nervous system cancer responsiveness to enediyne chemotherapy. *Cancer Chemother Pharmacol* 62: 699-706, 2008.
29. Kasimir-Bauer S, Ottinger H, Meusers P, Beelen DW, Brittinger G, Seeber S and Scheulen ME: In acute myeloid leukemia, coexpression of at least two proteins, including P-glycoprotein, the multidrug resistance-related protein, bcl-2, mutant p53, and heat-shock protein 27, is predictive of the response to induction chemotherapy. *Exp Hematol* 26: 1111-1117, 1998.
30. Walter RB, Raden BW, Kamikura DM, Cooper JA and Bernstein ID: Influence of CD33 expression levels and ITIM-dependent internalization on gemtuzumab ozogamicin induced cytotoxicity. *Blood* 105: 1295-1302, 2005.
31. Jedema I, Barge RM, van der Velden VH, Nijmeijer BA, van Dongen JJ, Willemze R and Falkenburg JH: Internalization and cell cycle dependent killing of leukemic cells by gemtuzumab ozogamicin: rationale for efficacy in CD33-negative malignancies with endocytic capacity. *Leukemia* 18: 316-325, 2004.
32. Van der Velden VHJ, Boeckx N, Jedema I, te Marvelde JG, Hoogeveen PG, Boogaerts M and van Dongen JJ: High CD33-antigen loads in peripheral blood limit the efficacy of gemtuzumab ozogamicin (Mylotarg) treatment in acute myeloid leukemia patients. *Leukemia* 18: 983-998, 2004.