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The biology of MDR1-P-glycoprotein (MDR1-Pgp) in designing functional antibody drug conjugates (ADCs): the experience of gemtuzumab ozogamicin

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Abstract

Background. The treatment of cancer remains a formidable challenge owing to the difficulties in differentiating tumor cells from healthy cells to ameliorate the disease without causing intolerable toxicity to patients. In addition, the emergence of MDR1-Pgp mediated multi-drug resistance (MDR) it is a biological phenomenon that inhibits the curative potential of chemotherapeutic treatments. One way to improve the selectivity of therapeutic molecules in tumors would be to target them on the tumor site, thereby sparing normal tissues.

Aims. In this overview, we will discuss the biological factors influencing the safety and efficacy of the humanized mAb hP67.6 linked to the potent cytotoxic drug calicheamicin-gamma1 (gemtuzumab ozogamicin) that target CD33 cell surface antigen expressed on AML cells. In addition, we highlight key aspects of MDR1-Pgp biology as a platform to understand its functional role in gemtuzumab ozogamicin immunotherapy which is tightly linked to an accurate assessment of the MDR status of AML cells.

Discussion. Several factors may affect the efficacy and safety of immunoconjugates. These include the common issues of chemical and antibody therapeutics such as specificity, heterogeneous target antigen expression and the complex pharmacokinetics profile of conveyed antibody. Further, the delivered drug may not be sufficient for providing therapeutic benefit, since the curative cytotoxic compound may be affected by intrinsic or acquired resistance of target cells. These and other potential problems, as well as the possible ways to overcome them will be discussed in this review by examining the biological factors involved in safety and efficacy of the first in class antibody drug conjugate (ADC) gentuzumab ozogamicin. Despite this set-back, the extensive recorded data and the lessons learned from gentuzumab ozogamicin recently withdrawn from the market for safety concerns helped to pave the way for next generations of clinically promising new ADCs which are currently investigated in clinical trials and two of them, Brentuximab vedotin, and Trastuzumab emtansine (T-DM1) have been recently approved for commercial distribution in US by Food and Drug Administration (FDA).

Key words
- antibody drug-conjugates
- gemtuzumab ozogamicin
- multidrug resistance
- MDR1-P-glycoprotein
- AML
- MDR reversing agent
- monoclonal antibody

INTRODUCTION

For decades combination chemotherapy with surgery or radiotherapy has become standard practice in the treatment of tumors and the use of these multimodal treatments has increased the survival rate of patients. One of the most recent improvements in the cure rate of tumor patients is achieved by dose-intensification, therefore paying the price of severe toxicity and high rate of life-threatening late events, such as secondary malignancies. This poses serious questions about quality of life and their long life expectancy. Little progress is achieved in improving survival for several common cancers such as lung, colon cancer or the less frequent melanoma; therefore, it is generally acknowledged that the benefits from chemotherapy in these diseases have reached a plateau and new therapeutic strategies are urgently needed [1, 2]. The development of selective and better tolerated cancer therapeutics represents an important goal in the research of new and more effective

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tumor treatment. Antibody-based cancer therapies have given promising results in several malignancies and specific monoclonal antibodies (mAbs), have demonstrated clinical efficacy even in patients with tumors only partially responsive to chemotherapy [3]. Although it seems unlikely to cure tumor mass by the administration of mAbs alone, the combination of mAbs exerting effector function (*i.e.*, apoptosis) with conventional anticancer drugs represents an effective strategy to overcome the intrinsic or acquired resistance of tumors which often are extremely aggressive and show a low survival rate despite the adoption of multimodal treatments [4]. Combining different cytotoxic drugs is a widely and successfully used clinical strategy that increases the response rate and duration of individual expectancy of life. The use of antibodies in conjunction with chemotherapeutics is a natural extension of this approach, and is strongly supported by preclinical studies that show improved efficacy of antibody and chemotherapeutic combinations compared with each drug used in isolation. For example, Herceptin has synergistic anti tumor activity when used in combination with cisplatin and carboplatin [5, 6] and additive benefit when used in conjunction with doxorubicin, cyclophosphamide, methotrexate, taxol or the selective cyclooxygenase-2 inhibitor, celecoxib [7, 8]. The addition of Herceptin combined with cytotoxic chemotherapy regimen was associated with statistically significant benefits in a Phase III trial in *ERB2*-overexpressing metastatic breast cancer. These gains included longer median duration of response (9.1 *vs* 6.1 months), higher overall response rate (50% *vs* 32%) and lower death rate at one year (22% *vs* 33%) [9]. In this overview, we will discuss the biological factors influencing the safety and efficacy of the humanized mAb hP67.6 linked to the potent cytotoxic drug calicheamicin-gamma1 (gemtuzumab ozogamicin) targeting CD33 cell surface antigen expressed on acute myeloid leukemia (AML) cells. In addition, we highlight key aspects of MDR1-Pgp biology as a platform to understand the functional role of gemtuzumab ozogamicin-treated AML patients and the clinical relevance of an accurate assessment of the MDR status of cellular malignancy. To this regard a particular emphasis is placed on the utilization of selected mAbs and staining methodologies to define a reliable expression level of MDR1-Pgp in young and elderly patients affected by AML.

ANTIBODY DRUG CONJUGATES AS THERAPEUTICS

Since the use of mAbs as single agents is sub-optimal, many strategies to improve efficacy are being investigated, including genetics or biochemical conjugation with cytotoxic drugs and toxins. Covalent conjugation of mAbs with drugs is not a new concept. Antibody drug conjugates (ADCs) comprise an antibody (or antibody fragments) conjugated with a cytotoxic drug via chemical linker. The therapeutic concept of ADCs is to use an antibody as a vehicle to deliver the cytotoxic drug to the tumor cell surface antigen thus sparing normal tissues. As a consequence, ADCs have significant potential for enhancing the antitumor activity of 'naked' antibodies and reducing the systemic toxicity of the cytotoxic drugs [10]. The concept of ADCs evolved from the hope that targeted delivery with mAbs would confer a degree of tumor selectivity to approved anticancer drugs such as doxorubicin, methotrexate, mitomycin-C, 5-fluorouracil and vinca alkaloids thus improving their therapeutic index [11-14]. These early conjugates were explored in human clinical trials but had limited success due to lack of potency. The lessons learned from these early explorations led to improvements in essentially all aspects of antibody conjugate therapeutics and hence to renewed interest in ADCs technology [15, 16]. On the other hand, there are highly cytotoxic compounds available that are too cytotoxic for direct application in the clinic due to their lack of selectivity. The basic idea is that over expression of the antigen on the corresponding tumor leads to an adequate selectivity. Upon uptake by the tumor tissue or cell, the ADC is cleaved to release the cytotoxic agent either within or in proximity to the tumor. The decomposition of the ADC and release of the cytotoxic moiety is most often triggered by specific enzymes and/or by the different pH in defined cellular compartments [17, 18]. The results with low potency ADCs prompted significant efforts towards utilizing drugs with much higher potencies which include calicheamicin, maytansine and auristatin (free drug potency around 10-9 M, 10-11 M) [19]. Pre-clinical studies have clearly shown that incorporation of highly potent drugs to the antibodies results in more effective reagents than using low potency drugs already studied for cancer therapy (free drug potency around 10-7 M) [20].

GEMTUZUMAB OZOGAMICIN AND RECENTLY DEVELOPED ADCs FOR AML IMMUNOTHERAPY

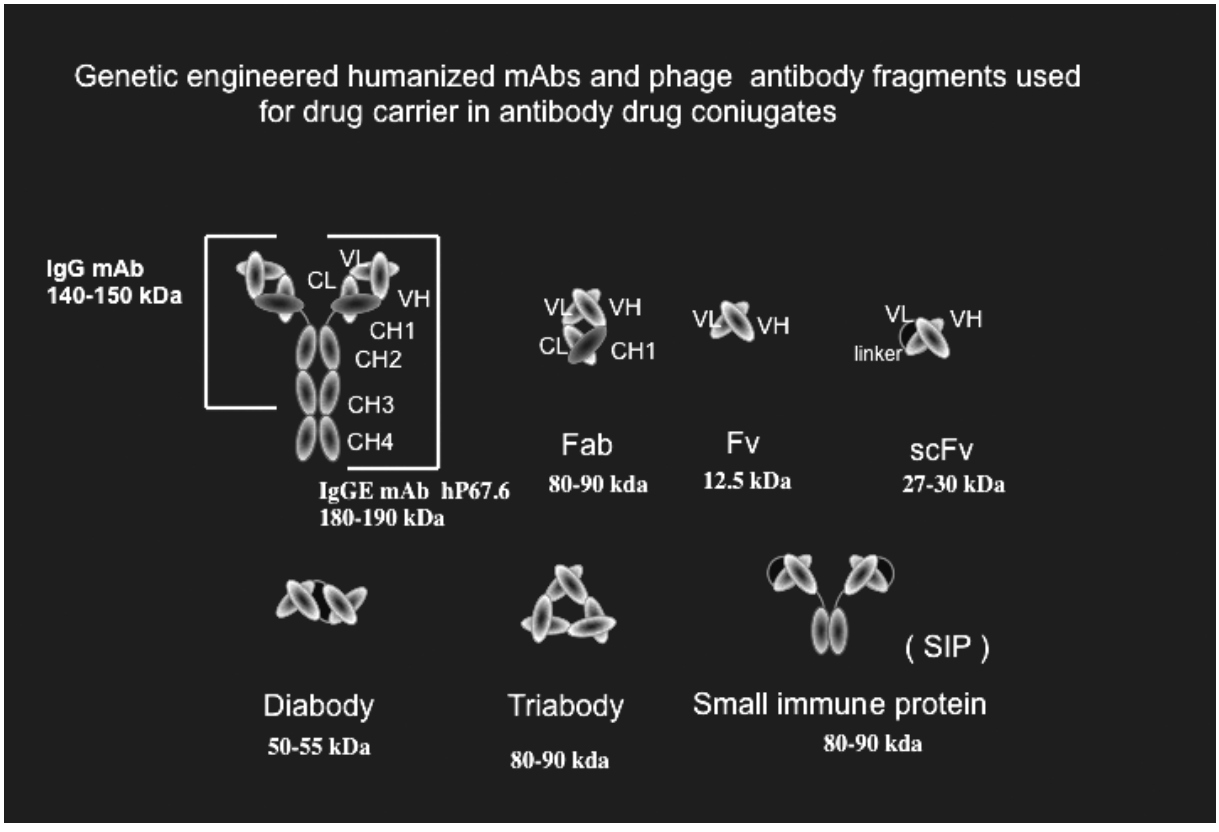
As the result of such improvements, gemtuzumab ozogamicin (Mylotarg) became the first ADC to be approved by the US Food and Drug Administration (FDA) for the treatment of AML [21]. This first in class ADC was withdraw from the market in June 2010 for the severe side effects emerging after a decade of pharmacological surveillance and *ad hoc* designed clinical trials [22]. Nevertheless, the technical and biological challenges encountered in designing gemtuzumab ozogamicin were the source of a biotechnological platform which is continuously being expanded for the development of an asset of ADCs which are genetically and biochemically designed to improve the safety and efficacy of this novel immunotherapeutic strategy against cancer. Recently, US FDA approved SGN-35, Brentuximab vedotin [23]. The drug consists of a mAb targeting CD30 linked to monomethyl auristatin E, a highly potent antitubulin agent. In addition, T -DM1, a trastuzumab-emtansine [24] and inotuzumab ozogamicin, an anti-CD22 con-

jugated with calicheamicin [25] are in advanced state of clinical evaluation for the treatment of breast cancer resistance to trastuzumab and for refractory and relapsed acute lymphocytic leukemia (ALL), respectively. Further, the optimization of linkers that couple the drug to the antibody providing sufficient stability of the antibody-drug conjugate in the circulation and optimal activation of the drug in the tumor, pave the way for designing novel generations of ADCs most of them are in advanced phases of pre-clinical and clinical studies for treatment of different diseases with unmet medical needs [26, 27]. However, several factors may affect the efficacy of ADCs. These include the common issues of mAb therapy such as antibody specificity, low and heterogeneous target antigen expression and large inter-individual differences in cellular sensitivity of tumor cells to drugs [28]. A series of concerns about the safety and efficacy resulted during clinical utilization of gemtuzumab ozogamicin. For example, the cytotoxicity observed *in vivo* during gemtuzumab ozogamicin treatment in non CD33 cells is a clear evidence that the acid-labile hydrazone linker broke down in the blood stream allowing the drug to strike normal tissues and organs. Therefore, it is possible that non-specific drug release through linker instability contributes to the activities of gemtuzumab ozogamicin in non CD33 tumor cell types and affecting physiological function of normal tissues and cells causing severe toxicity [19, 20]. In addition, sub-lethal concentrations of the mAb conjugate may create the biological conditions driving for selection of tumor cell variants with lower CD33 target expression or altered hP67.6 epitope conformation no longer recognized by the specific antibody ligand [29].

Theoretically, the ADC offers highly selective and very effective anti-tumor strategy, and has been pursued for more than two decades. Still, its translation into clinical practice has suffered from several drawbacks. To this regard, it should emphasize that cytotoxic compounds used in several ADCs formulation such as calicheamicin may be substrates for the MDR1-Pgp multidrug transporter, and the clinical success of this new concept of immunotherapy it is functionally linked to the intrinsic or acquired expression of multidrug resistance (MDR) in target tumor cells [30-32]. Reports linking overexpression of the MDR1-Pgp to adverse outcome in adult AML patients [33, 34] provided the evidence necessary to implicate the MDR phenotype as an important biologic factor to be considered, i) for modeling appropriate

Figure 1

Schematic conformational structure of recombinant monoclonal antibodies. The different immunocompetent molecules currently used to deliver cytotoxic drugs to target tumor cells are obtained by humanized antibodies generated by the insertion of mouse complementarity-determining regions (CDRs) onto human constant and variable domain frameworks (IgG, IgGE). Fully human antibodies can be also generated by the selection of human antibody fragments from *in vitro* libraries, by transgenic mice and through selection from human hybridomas. Single chain fragment variable (scFv) antibodies and derived antibody structures reported in the figure are obtained by phage display antibody library using different biochemical linker



immunochemotherapy regimen still effective in presence of MDR1-Pgp expression and, ii) to investigate the biological role of MDR1-Pgp and related multidrug transporter proteins in the safety and efficacy of AML cells during induction, consolidation and maintenance/post remission therapeutic treatments.

INNOVATIVE MEDICAL APPROACHES FOR AML TREATMENT

AML represents a group of clonal hematopoietic stem cell disorders in which both failure to differentiate and over proliferation in the stem cell compartment result in accumulation of non-functional cells termed myeloblasts. The standard treatment paradigm for AML is remission induction chemotherapy with an anthracycline/cytarabine combination, followed by either consolidation chemotherapy with high or intermediate doses of cytarabine, aims at reducing the undetectable burden of leukemic cells to a level low enough that long term disease- free survival might be possible. The curative regimen may include haematopoietic stem cell transplantation (HSCT), depending on the patient's ability to tolerate intensive treatment and the likelihood of cure with chemotherapy alone. Although this approach has changed little in the last three decades, increased understanding of the pathogenesis of AML and improvements in molecular genomic technologies are leading to novel drug targets and the development of personalized, risk-adapted treatment strategies [35, 36]. With standard induction chemotherapy, complete remission is obtained in nearly 70% of the patients. For consolidation therapy, the most effective leukemia treatment is HSCT. However, this medical intervention carries a high risk of initial mortality and a significant risk of long-term morbidity associated with chronic graft-versus-host disease, which tends to offset therapeutic benefits of a low likelihood of relapse [37]. Chemotherapeutic-based approaches can be performed relatively safely, but the risk of disease recurrence remains high and patients with relapsed AML have a particularly poor prognosis [38, 39].

A number of cytotoxic agents and combination regimens have been used for salvage chemotherapy and remission rates from 20-70% are described. However, the period of remission lasts only typically between 4-6 months. In addition, chemotherapy is accompanied by substantial side effects because the cytotoxic drugs not only kill leukemic cells but also dividing cells of hematopoietic lineages. Therefore, it is clear that new additional therapy approaches are required. A promising therapy is antibody-targeted therapy, in which the effector function of cytotoxic drug as well as destructive radioisotopes are tumor delivered by specific antibody [40, 41]. In AML, the CD33 antigen is an appropriate target because AML blast cells express the CD33 antigen in 80-90% of patients, whereas hematopoietic stem cells, lymphoid cells, and several other tissues do not express the CD33 antigen which is a membrane-bound glycoprotein of 67 kDa belonging of the immunoglobulin superfamily [42]. ADCs show efficacy over a wide range of antigen expression levels. For example, while CD33-positive AML tumors express relatively low levels of target antigen (5000-10 000 receptors per cell) [43] meaningful clinical responses have been observed with gemtuzumab ozogamicin. Effective treatment of tumors with such low antigen expression levels requires an ADC with a highly potent toxic component, such as calicheamicin. An alternate approach using specific anti CD33 mAb to kill AML cells it is also attempted. In this context, the first immune radiolabeled curative investigation was conducted with mAb195 in its original murine form [44]. Subsequently, the genetically engineered humanized version designated Hum195 and also known as Lintuzumab or SGN-33 is entered in various phases of clinical trials in combination with different chemotherapy regimen [45-49] and IL2 [50]. To this regard, Lintuzumab demonstrated significant anti-tumor activity through its ability to mediate effector functions and to engage intracellular signaling processes associated with decreased production of tumorigenic and immunosuppressive factors. These activities may also promote the development of anti-tumor immune responses and correlate well with the significantly prolonged survival that was observed in preclinical models of AML. These data clearly show that Lintuzumab is a valid, targeted therapeutic, the activity of which is not affected by common mechanisms of MDR and related drug transporter proteins. Furthermore, this anti CD33 antibody in its various therapeutic combinations represents a potential treatment option for AML patients, especially those unable to tolerate high-dose induction chemotherapy [45-50]. This clinical option may be of particular medical relevance in the current situation where the gemtuzumab ozogamicin is no more available for new AML patients [22] while the recently positive responses in re- modulated curative regimen are not yet considered by governmental institutions for clinical re-assessment of calicheamicin-gamma1 anti CD33-linked antibody [51-53].

THE BIOLOGY OF GEMTUZUMAB OZOGAMICIN

mAbs represent the fastest growing sector of pharmaceutical biotechnology but, in many cases the antibodies used for the treatment of tumors offer only a modest survival benefit to cancer patients thereby yielding marginal therapeutic indices. While non modified mAbs used as single agent for cancer treatment are active, they are rarely curative [1-4]. As a result, considerable attention has turned in enhancing antibody activity by appending cytotoxic drugs to them generating ADCs capable to site-selective drug delivery. The rationale for the development of ADCs, is to combine the selectivity, favorable pharmacokinetics, bio-distribution and, when present, functional activity of antibodies (*Figure 1*) with the high cytotoxic potency of a drug. An ideal ADC should: retain the

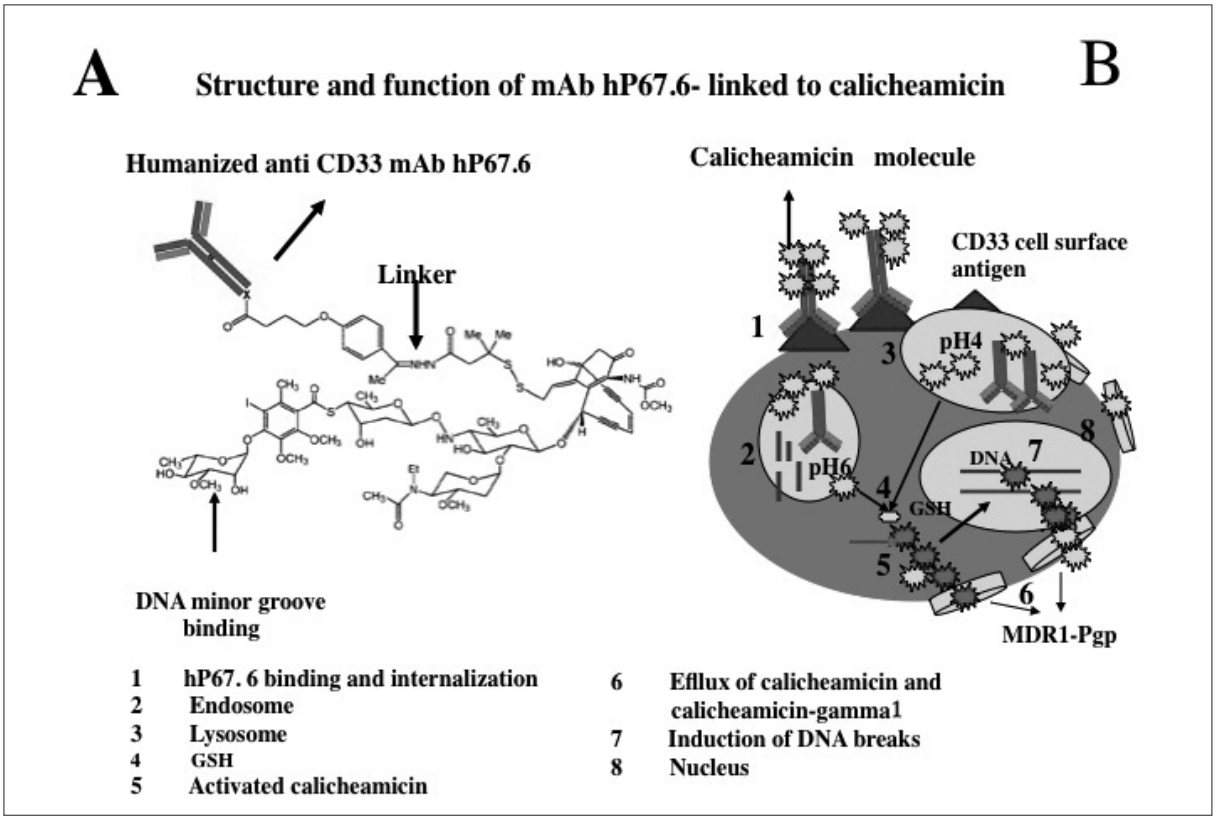


Figure 2
Structure and function of gemtuzumab ozogamicin (Mylotarg). In panel **A** the humanized IgG4 isotype monoclonal hP67.6 is conjugated to a cytotoxic agent N-acetyl gamma calicheamicin dimethyl hydrazide (NAc-gamma calicheamicin DMH) *via* the bifunctional AcBut linker. In Panel **B**, The hP67.6 antibody –calicheamicin conjugate binds to the CD33 antigen, is internalised into lysosomes and endosomes where acidification releases the NAc-gamma calicheamicin DMH moiety. The latter undergoes spontaneous reaction with reduced glutathione (GSH) within the cell, where it is activated and the anti-tumour effect can occur. The anti-tumor mechanism is thought to occur by binding to the minor groove in the DNA and producing site-specific double-strand breaks by forming p-benzene diradical. The result is the death of the leukaemic cells

favorable pharmacokinetic and functional properties of antibodies; remain intact and non toxic in the compartment of its systemic delivery (typically blood); become active in the tumor compartment, with the active form of drug released in a sufficient amount to kill tumor cells. Other important key parameters in designing an effective ADCs are the choice of target antigen, the ability of the ADC to localized to target tissues, the fate of the antibody once bound to its cognate antigen, and the cytotoxic potency and selectivity of the released drug *vs* tumor cells [19, 20]. Older patients with AML are thought to have a poorer outcome than younger patients with AML because of biological differences in the disease and because of the inability of older patients to tolerate more intensive anti leukemic therapies. The disease in older patients commonly evolves from previous hematologic disorders, such as myelodysplastic syndrome (MDS), which is often refractory to therapy [54]. Older AML patients have a higher incidence of poor- risk cytogenetic abnormalities (*Table 1*) and expression of the multidrug resistance phenotype. In addition, they tolerate intensive chemotherapy poorly because of comorbid conditions and the decreased ability to tolerate myelosuppression [55]. Thus, treatment outcome is poor in older patients with AML, and for many, the goal is palliation of symptoms rather than cure. Antibody-targeted chemotherapy is expected to be less toxic than conventional chemotherapy and was developed for the treatment of CD33-positive AML [21]. Gemtuzumab ozogamicin consists of an anti-CD33 mAb conjugated with a derivative of calicheamicin, a highly potent enediyne antibiotic [56]. This ADC is a humanized (CDR-grafted) IgG4 isotype monoclonal antibody directed towards CD33 antigen that is conjugated to a cytotoxic agent N-acetyl gamma calicheamicin dimethyl hydrazide (NAc-gamma calicheamicin DMH) via the bi-functional Ac-Butlinker [57]. The hP67.6 antibody (non-cytotoxic by itself) binds to the CD33 antigen, and the antibody drug conjugated is internalized into lysosomes and endosomes where acidification releases the NAc-gamma calicheamicin DMH moiety.

The latter undergoes spontaneous reaction with re-

duced glutathione (GSH) within the cell, where it is activated and the anti-tumor effect can occur [58]. The active drug (thereafter referred as calicheamicin-gamma1) is a potent anti-tumor antibiotics that were initially identified by their ability to damage DNA in screening tests (*Figure 2*, panel A). Its anti-tumor mechanism is thought to occur by binding to the minor groove in the DNA and producing site-specific double-strand breaks by forming p-benzene di-radical [59]. This results is the death of the leukaemic cells (*Figure 2*, Panel B). Gemtuzumab ozogamicin was approved by the FDA, in US in May 2000 for the treatment of AML patients who suffer from a first relapse, are ≥ 60 years old and are not candidates for other cytotoxic chemotherapy [60]. The IgG4 isotype was chosen because it has the longest circulating half-life of all isotypes and is least likely to participate in immunomediated mechanisms such as complement fixation and antibody dependent cellular toxicity. Both rodent and humanized mAbs recognized the identical epitope localized in external domain of the cell surface of CD33 determinant. The mAb hP67.6-calicheamicin-gamma1 conjugated drug substance is a heterogeneous mixture of 50% conjugated (0 to 8 calicheamicin-gamma1 moieties per IgG molecule, with an average of two or three, randomly linked to solvent-exposed lysyl residues of the antibody) and 50% unconjugated antibody [21]. Despite encouraging clinical results that led to accelerated approval of gemtuzumab ozogamicin in 2000, the completion of the ongoing studies of gemtuzumab ozogamicin in relapsed AML and initiation of randomized clinical trials comparing the effects of gemtuzumab ozogamicin in combination with conventional induction chemotherapy to conventional chemotherapy alone on survival are mandated to confirm clinical benefit under the accelerated approval Subpart H regulations. However, as reported in the approval summary [21] several factors may affect the safety of gemtuzumab ozogamicin. Postmarketing reports of fatal anaphylaxis, adult respiratory distress syndrome (ARDS), and hepatotoxicity, especially veno occlusive disease (VOD) in patients treated with gemtuzumab ozogamicin, with and without associated hematopoietic stem cell transplantation (HSCT), have required labeling revisions and the initiation of a registration surveillance program. Tumor lysis and ARDS have been reported in patients with leukocytes above 30 000 / ml treated with gemtuzumab ozogamicin; therefore, the reduction of leukocyte counts to below 30 000 / ml is recommended prior to treatment. Patients should be carefully monitored for acute hyper sensitivity, hypoxia, and delayed hepatotoxicity following treatment with gemtuzumab ozogamicin. Hence,a subsequent Phase III confirmatory trial raised new concerns about the product's safety and failure to demonstrate clinical benefit [22]. Subsequent findings in three additional randomized trials comparing standard induction chemotherapy with and without gemtuzumab ozogamicin in newly diagnosed AML patients [51-53] stood in contrast to the Phase III confirmatory study and suggested clinical benefit among certain patients -those whose AML was characterized by either good or intermediate cytogenetics risk (*Table 1*) [61]. The impact that the different Phase III trials might have on the future development of gemtuzumab ozogamicin as a drug remains for global regulatory bodies to determine. Several lessons can be taken from the gemtuzumab ozogamicin development program. Calicheamicin is hydrophobic, and only a few drugs can be conjugated before high levels of aggregated protein are obtained. Consequently, the manufacturing process used at the time gemtuzumab ozogamicin was developed yielded 50% unconjugated mAb in the final drug product [21]. The linker between calicheamicin and the mAb released 50% of bound drug in 48 h while the half-life of the ADC is 67 h, and a 2-week interval between doses of (9 mg/m2) was chosen to prevent drug accumulation. Pharmacokinetics studies in adults and children with AML demonstrated that the antibody conjugate concentrations increase after the second dose, probably because of reduced tumor burden [62]. Pharmacokinetics were similar in children and adults [63, 64]. Furthermore, novel ADC's targeting calicheamicin-gamma1 in CD22 positive haematological malignancies (ALL) have explored at the preclinical and clinical level for their curative potential [65, 66].

THE BIOLOGY OF MULTIDRUG TRANSPORTER AND ITS ROLE IN THE EFFICACY OF GEMTUZUMAB OZOGAMICIN

In principle, the biological entity playing a key role in the efficacy of gemtuzumab ozogamicin is represented by the overexpression of the multidrug transporter proteins which acting as an efflux pump may remove anticancer drugs from cells, resulting in a simultaneous cross-resistance or MDR to various chemotherapeutic agents which include calicheamicin-gamma1 as well as other cytotoxic compounds used for AML treatment [67, 68]. Inherent or acquired resistance of tumor cells to cytotoxic drugs represents a major limitation to the successful chemotherapeutic treatment of cancer. During the past three decades dramatic progresses were made in the understanding of the molecular basis of this phenomenon. Analyses of drug-selected tumor cells which exhibit simultaneous resistance to structurally unrelated anti-cancer drugs have led to the discovery of the human MDR1 gene product, MDR1-Pgp encoded by MDR1/ABCB1 gene localized to 7q21 as one of the mechanisms responsible for MDR [30-32, 69]. Furthermore evidence from *in vitro* studies of primary AML blasts supports the commonly held supposition that MDR1-Pgp expression may be linked to apoptosis-resistance. This phenomenon that significantly interfere with functional activity of gemtuzumab ozogamicin may

be biologically linked with phenotypic alteration of the presence of MDR1-Pgp in AML cells which include modulation of cytokine efflux, signalling lipids and intracellular pH [70].

The key role of MDR1-Pgp in derivative calicheamicin-gamma1 antibody drug conjugate is also confirmed by its remarkable activity in relapsed acute promyelocytic leukemia (APL). This haematological malignancy combines a high and homogeneous expression of the gemtuzumab ozogamicin target antigen CD33 with low levels or absence of MDR1-Pgp [71]. Gemtuzumab ozogamicin shows prolonged molecular remissions in APL both as a single agent and in combination with specific drugs. In addition to efficacy, gemtuzumab ozogamicin curative regimen in APL is associated with good safety profiles since heavy adverse effects such as VOD observed in AML treatment were not observed in APL [71]. Decades of research have emphatically demonstrated that AML differs widely both clinically (most notably in response to standard treatments) and in molecular, genetic, and epigenetic characteristics [72]. Furthermore, bioinformatic approaches, taking advantage of large drug databases tested across well-characterized cell lines, have allowed the identification of several potential cytotoxic substrates recognized by different ABC transporters [73, 74]. In addition, pharmacokinetics analyses and the study of knockout mice have revealed important roles of several ABC transporters in the absorption, excretion and distribution of drugs. ABC transporters are essential for many cellular processes that require the transport of substrates across cell membranes [75]. Clinical trials aimed to link MDR1-Pgp expression with poor clinical outcome was legitimate in breast cancer, sarcoma and certain types of leukaemia, because MDR1-Pgp positive patients with these cancers were compared with MDR1-Pgp-negative patients of the same cancer type. These findings overviewed by Szakács *et al.*, [31] report leading examples such as, a meta-analysis of 31 breast cancer trials showed a threefold reduction in response to chemotherapy among tumors expressing MDR1-Pgp after treatment [76]. In another study, MDR1-Pgp was found to be expressed in as many as 61% of pre-treatment soft tissue sarcomas (STS); even higher expression occurred following therapy with doxorubicin [77]. This is likely to be clinically important as doxorubicin is a known MDR1-Pgp substrate and one of the main chemotherapeutic agents commonly used to treat STS. However, the validity of these findings remains controversial as MDR1-Pgp positivity was variably defined throughout the trials, a limitation that is inherent to numerous studies assessing the impact of MDR1-Pgp expression on patient survival. In contrast to solid tumors, haematological malignancies are much easier to collect and purify. This relative cellular homogeneity has allowed a more reliable determination of MDR1-Pgp expression in leukaemic cells using techniques such as immuno-flow cytometry and reverse transcription-polymerase chain reaction (RT-PCR) [78]. Functional assays, such as those using flow cytometry to measure efflux of fluorescent MDR1-Pgp substrates (for example, calcein-AM and rhodamine-123) from leukaemic cells, often complement MDR1-Pgp expression analysis [79, 80].

Using these techniques, more than a third of leukaemic samples are found to be positive for MDR1-Pgp expression, and so the adverse impact of MDR1-Pgp expression on patient survival or response rate has been most comprehensively evaluated for haematological malignancies, particularly AML and MDS. MDR1-Pgp expression in patients with AML has consistently been associated with reduced chemotherapy response rates and poor survival, and it was found to be an independent prognostic variable for induction failure in adult AML [34, 81]. The functional role of MDR1-Pgp offers the rationale for using drug efflux inhibitors to enhance gemtuzumab ozogamicin-induced cytotoxicity, thereby possibly improving clinical outcome of patients undergoing gemtuzumab ozogamicin-containing AML therapy [82].

EXPRESSION OF ABC TRANSPORTERS AND AML PHENOTYPIC ALTERATION DURING DRUG TREATMENT

Although MDR1-Pgp appears to be of biologic and clinical relevance, other ABC proteins have been hypothesized to be involved in the emergence of multidrug transporter in cancer as survival mechanism over expressed in adverse selective environment. One such protein, MRP1 is distantly related to MDR1-Pgp, and like MDR1-Pgp, lowers intracellular drug accumulation by promoting drug efflux and MDR [83]. Previous studies hypothesized an association between MDR1-Pgp and MRP1 expression and clinical responses to gemtuzumab ozogamicin [84], others reported discrepant results attributed to the multi-factorial nature of drug resistance [30-32]. Engagement of CD33 by gemtuzumab ozogamicin results in ADC internalization and hydrolytic release of the toxic calicheamicin-gamma1 moiety which causes DNA damage and cell death [21]. Even tough, CD33 expression and related pathways involved in gemtuzumab ozogamicin induced cytotoxicity are the object of several studies, the resistance mechanism emerging from CD33-gemtuzumab ozogamicin interaction is not yet fully understood. In order to elucidate the cellular factors contributing to free and mAb linked calicheamicin-gamma1 resistance a panel of HL60 MDR cells was created *in vitro* system and used to analyze a series of biological factors tightly linked with gemtuzumab ozogamicin efficacy [85]. These include: i) the ability of calicheamicin-gamma1 to interact with the MDR1-Pgp and MRP1 drug efflux systems expressed in MDR variants, ii) the role of MDR1-Pgp and MRP1 in conferring free and mAb linked calicheamicin-gamma1 resistance and nature of cell death in calicheamicin-gamma1 induced cytotoxicity,

Table 1
Cytogenetic risk groups and molecular abnormalities

Favorable	Intermediate	Adverse
t(8;21) t(15;17) inv(16) Normal cytogenetics with NPMI mutation or CEBPA mutation in absence of FLT3-ITD mutation	Normal +8 +21 +22 del(7q) del(9q) Abnormal 11q23 and all other structural/ numerical Abnormalities t(8;21), inv (16), or t(16;16) with c-KIT mutation	-5 -7 del(5q) Abnormal 3q Complex Normal cytogenetics with FLT3-ITD mutation

FMS-like tyrosine kinase 3 (FLT3) is a tyrosine kinase receptor with important roles in hematopoietic stem/progenitor cell survival and proliferation. It is mutated in AML patients, either by internal tandem duplications (ITD) of the juxtamembrane domain or by point mutations usually involving the kinase domain; NPM1, nucleophosmin gene CCAAT/enhancer binding protein α, (CEBPA). Mutation of the CEBPA may play an important role in leukemogenesis and prognosis.

and iii) the effect of gemtuzumab ozogamicin treatment on CD33 expression and its role in gemtuzumab ozogamicin resistance. The results obtained, strongly suggest that both MDR1-Pgp and MRP1 efflux systems are engaged by calicheamicin-gamma1, but only MDR1-Pgp overexpression efficiently abrogated calicheamicin-gamma1 drug cytotoxicity. In addition, calicheamicin-gamma1 exerts potent cytotoxicity via necrosis or apoptosis [85]. Moreover, at least in our experimental conditions, the CD33 down-modulation represents an important escape mechanism of HL60 cells from the cytotoxic effect of gemtuzumab ozogamicin. Both MDR1-Pgp and MRP1 expression are involved in drug transport and may determine, in adults suffering from AML treated with gemtuzumab ozogamicin the amount of calicheamicin-gamma1 that is available in the leukemic cells to induce apoptosis [70]. The tight relationship between expression of one or both of these ABC transporters and adverse outcome of gemtuzumab ozogamicin monotherapy is proposed on the basis of clinical investigation and/or *ex vivo* analysis of AML blast samples [84]. In contrast, very few studies have been conducted to verify as to whether MDR1-Pgp and MRP1 expression confer calicheamicin-gamma1 resistance in *in vitro* conventional approach consisting of the comparison of sensitive cells with drug resistant counterpart that were experimentally induced to become MDR.

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 [30-32]. 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-overexpressed [86, 87]. 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 selected in presence of escalating dose of doxorubicin. By using mAbs MM4.17 [88] and MRP1m6 [89] 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 positive. Afterwards, MRP1 over-expression is observed after a first step of selection coincident with the isolation of HL60 with an intermediate level of drug resistance. A subpopulation of these MDR variants reacted with the mAb MM4.17 confirming that MDR1-Pgp and MRP1 may be co-over-expressed. Finally, HL60 MDR cells are characterized by a very high level of MDR1-Pgp and abrogation of MRP1 expression. These findings indicate that the HL60 cell system we used, is an appropriate *in vitro* approach to study the correlation between MDR phenotype and free or mAb linked calicheamicin-gamma1. To this regard, studies conducted in our laboratory using the above described *in vitro* model constituted by a series of sensitive/resistant HL60 cell pairs shows that drug efflux mediated by MDR1-Pgp results in resistance to gemtuzumab ozogamicin; conversely, the expression of MRP1 does not affect at least in *in vitro* system gemtuzumab ozogamicin -induced cytotoxicity [85]. The existence of a quantitative relationship between CD33 expression and *in vitro* response to gemtuzumab ozogamicin 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 (90). 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 responders, with CD33 expression and MDR1-Pgp activity exhibiting an inverse correlation [34]. In contrast, current experience indicates, however, that treatment failure or drug resistance to gemtuzumab ozogamicin is not commonly associated with outgrowth or selection of CD33 -negative leukaemia. In spite of these important questions, little efforts are currently applied to study the mechanism gemtuzumab ozogamicin resistance in a classical *in vitro* approach, namely the exposure of AML cells to sub-effective dose gemtuzumab ozogamicin and verifying after an extended period of time as to whether, the phenotypic alteration of the CD33 may be related with an escape of antibody-mediated cytotoxicity. To this regard, we exposed HL60 cells to a gemtuzumab ozogamicin dose of 5 ng/ml that killed 80-90% of the cells in the first round of treatment and the surviving 10-20% of the cells were maintained in an uninterrupted subculture with 5 ng/ml of gemtuzumab ozogamicin for 8 weeks. As control, in parallel culture HL60 cells were treated with identical modality but in presence of only the anti-CD33 drug-free mAb hP67.6. Finally, a gemtuzumab ozogamicin resistant cell line named HL60/GO was created and investigated for CD33 expression and susceptibility to free- or mAb hP67.6 linked calicheamicin-gamma1. The results show that the HL60/GO cell line has a significant reduction of mAb binding, and in comparison with the parental HL60 cells untreated or treated with only mAb hP67.6 appear to be more resistant to gemtuzumab ozogamicin. The linkage between CD33 mAb hP67.6 binding and gemtuzumab ozogamicin susceptibility was also demonstrated in revertant cells isolated by culturing (for two weeks) HL60/GO-resistant cells in gemtuzumab ozogamicin free-medium. These cells regain the standard CD33 expression level and gemtuzumab ozogamicin susceptibility. In addition, the selective pressure utilized for the isolation of gemtuzumab ozogamicin resistant variants does not modulate MDR1-Pgp and MRP1 expression or drug efflux function. These findings indicate that CD33 down-modulation may represent an efficient escape mechanism for AML treated with (sub-effective dose) of gemtuzumab ozogamicin. Nonetheless, important questions raised about the role of CD33 in gemtuzumab ozogamicin mediated cytotoxicity remain unresolved and further studies are warranted. In conclusion, by this investigation aimed to analyze the modulation of biological MDR phenotype under the selective pressure of calicheamicin gamma1 we demonstrated that CD33 down modulation represents an efficient drug resistance mechanism as response to gemtuzumab ozogamicin treatment. In conclusion, expression and modulation of MDR1-Pgp and CD33, respectively represents two biological factors playing a key role in the efficacy of calicheamicin-gamma1. However, it is cannot ruled out that gemtuzumab ozogamicin resistance may originate by decreased accessibility of the hP67.1 calicheamicin-gamma1-linked antibody to CD33 cell surface antigen masked by newly over-expressed membrane proteins harboring in cell variant by the selective culture condition treatment [29].

MDR1-Pgp AND PROGNOSTIC FEATURES

In recent studies aimed to reassess gemtuzumab ozogamicin as a medically relevant option for AML treatment in its different pathological manifestations, the prognostic features of the MDR1-Pgp expression level is completely abrogate and relegated as control of MDR phenotype in a fraction of patients included in the AML15, AML16 and ALFA-0701 trials [51-53]. The investigators assuming that the approved dose (9 mg/m2) resulted in an excess of toxicity re-modulate dosage (3 mg/m2) and schedule of gemtuzumab ozogamicin administration obtaining excellent positive results [62]. In these studies the cytogenetics profile remains the most important feature for the clinical prognosis. Three risk categories -favorable, intermediate and poor risk- have been recognized based upon outcomes by chromosomal abnormalities in several large series of patients that differently benefit of the novel designed clinical regimens [91, 92]. However, in the last few years, there have been several practice changing developments in the diagnosis and treatment of AML. The old favorable, intermediate, and poor prognostic categories, which were based on cytogenetic risk groups, are no longer adequate. Advances in genomics technologies have identified AML as a genetically highly heterogeneous disease, and an increasing number of AML patients can now be categorized into distinct clinic pathologic sub-groups on the basis of their underlying molecular genetic defects. Cytogenetically normal patients, who comprise the largest subgroup and have historically been assigned as an intermediate prognosis, can now be further divided into a myriad of molecular subgroups, some of which are of relevant prognostic implications [93]. Despite substantial progress in the treatment of newly diagnosed AML, 20% to 40% of patients do not achieve remission with the standard induction chemotherapy, and 50% to 70% of first CR patients are expected to relapse within 3 years. The optimum strategy at the time of relapse, or for patients with the resistant disease, remains uncertain. Although several new agents have shown promise in treating AML [94], it is unlikely that these agents will be curative when administered as monotherapy; it is more likely that they will be used in combination with other new agents or with conventional therapy [95, 96]. Patients often relapse with unresponsive disease after an initial response to treatment with standard chemotherapeutic treatment. MDR1-Pgp is believed to function as an energy-dependent, efflux pump resulting in a decrease in intracellular drug concentrations to sublethal dosages.

Furthermore MDR1-Pgp expression proved to have independent influence on overall survival despite the correlation with high-risk cytogenetic aberrations [97].

Therefore, MDR1-Pgp over expression may not only be a secondary event activated by specific aberrations in cytogenetically unstable AML patients but seems to have additive effects on prognosis [98]. Significant correlations of MDR1-Pgp expression and the appearance of the high-risk aberrations like del(7q), del(5q) or abn (3q) are reported in patients with a bad prognosis (*Table 1*). However, different opinions on the key role of MDR1-Pgp in the outcome of AML chemotherapy is also reported. To this regard more comprehensive evaluation of the prognostic significance of MDR1-Pgp expression in the context of contemporary, intensive chemotherapy protocols failed to demonstrate independent prognostic significance to MDR1-Pgp expression when evaluated in the context of other adverse prognostic factors such as cytogenetics. These studies have demonstrated that MDR genes are highly expressed in older patients and those with high-risk cytogenetics not providing additional, clinically useful prognostic information. Furthermore, evaluation of MDR genes in pediatric patients also failed to demonstrate prognostic significance. Sievers *et al*., [99] demonstrated a prevalence of 13% for the expression of MDR1-Pgp in a group of 130 pediatric AML patients treated in the CCG 2891 trial. However, the clinical outcomes of those with and without MDR1-Pgp expression were not different. Additional pediatric studies have demonstrated that MDR1-Pgp expression is not higher overall in patients with relapsed AML [100]. Although MDR expression may not be an independent prognostic factor, it may be a useful therapeutic target in the management of innovative AML therapeutic strategies. Several agents show to impair the function of proteins encoded by MDR genes, which may potentially sensitize the MDR cells to the therapeutic effects of the specific chemotherapy agents [101, 102]. In combination with conventional chemotherapy, such agents may augment response to chemotherapy (collateral sensitivity) and improve survival. In spite of these considerations the accurate assessment of MDR status and the corresponding measurable level of MDR1-Pgp drug efflux function may represent a prerequisite for designing novel curative regimens based on antibody drug conjugates and to combine cytotoxic therapy with MDR reversing agents down modulating the drug resistant phenotype of AML cells [103, 104].

CRITICAL ISSUES FOR A RELIABLE DETERMINATION OF MDR STATUS IN YOUNG AND ELDERLY PATIENTS

One of the most important prerequisite to understand the biological and clinical significance of MDR1-Pgp in AML firstly depends on its expression level and concurrent drug efflux function during the different phases of chemotherapy treatments. However, a reliable measurements of MDR1-Pgp is hampered by the methods and antibody used and sensitivity of the concurrent assays for the evaluation of functional drug efflux activity. To this regard, in contrast to elderly patients the studies of MDR1-Pgp expression in children has revealed a lower incidence of this multidrug transporter protein [99, 100]. The positivity staining of AML cells defined by mAb MRK-16 [105] was as more than 5% of cells, which was found in 14% of samples. However, only 1.7% of samples showed MDR1-Pgp positivity using a more traditional cut-off level of 20%. In contrast to this finding, MDR1-Pgp expression investigated by a different group using the same mAb MRK-16 (5 μg/mL) was found in 58 untreated pediatric AML samples (median, 88% blasts; range 69%-98%). Applying a 20% cut-off level, 56 of 58 samples stained MDR1-Pgp positive (median 67% of blasts were MRK.16 positive; range 20%-94%) [100]. These contrasting observations on MDR1-Pgp expression level in two independent studies on young patients using the identical mAb and staining procedures pose a series of concerns on the methodology used to determine this critical biological entity in AML therapy. In agreement with the considerations reported by both groups of investigators no likely explanation other than technical issues can be provided for these differences [99, 100]. Furthermore, these investigators are in agreement with the need to assess MDR1-Pgp status by different techniques, including the use of more effective and high affinity antibodies capable to intercept even very low level of MDR1-Pgp expression in AML blast cells. This controversial position on MDR1-Pgp expression has a dual scientific merits: i) to focus the discussion on the clinical relevance of multidrug transporter proteins in relationship to AML curative regimen and, ii) to raise the question on reliability of methodological approaches to determine expression and function of MDR1-Pgp in AML cells as a prerequisite to design appropriated curative strategies. In contrast, the absence of reliability in MDR1-Pgp detection and expression level in AML specimen has determined an increase awareness and low confidence with this biological entity in clinical setting moving the scientists in designing therapies based on more reliable markers such as cytogenetic profiles [106]. To this regard, decades of research have emphatically demonstrated that AML differs widely both clinically (most notably in response to standard treatments) and in molecular, genetic, and epigenetic characteristics [107]. The extreme heterogeneity in the latter is uniformly acknowledged to indicate that optimal management of AML necessitate the knowledge of its MDR status which include MDR1-Pgp and related multidrug transporter proteins. To this regard, the analysis of drug efflux and is a possible surrogate marker of response to gemtuzumab ozogamicin, with no remissions observed in patients whose leukemic blasts showed relatively elevated levels of drug efflux. Even though additional studies are warranted to determine whether the phenotype of low compared with high levels of drug efflux helps to define a *priori* which patients are more or less likely to respond to gemtuzumab ozogamicin [79, 80], large body of evidences links the MDR1-Pgp expression with poor response of AML to pharmacological treatments [30-32, 69].

ASSESSMENT OF MDR STATUS OF AML BY USING SPECIFIC MABS TO EXTERNAL MDR1-Pgp

AML cells differ widely both clinically (most notably in response to standard treatments) and in molecular, genetic, and epigenetic characteristics. The extreme heterogeneity in the latter is uniformly acknowledged to indicate that optimal management of AML will eventually encompass many specific regimens that may be designed according to recognized biological and functional characteristics. Among these a pivotal role is played by MDR1-Pgp which may confer to AML multidrug resistance to calicheamicin-gamma1 delivered by anti CD33 antibody and the current drugs notably anthracyclines used in AML induction/consolidation therapies. A prerequisite to confer a prognostic factor and biological and clinical significance to MDR1-Pgp in the chemotherapy plus gemtuzumab ozogamicin regimen is irrespective of the absolute reliability and confidence of its MDR status. A number of impressive publications have shown that MDR1-Pgp's substrate specificity and mechanism of export are more sophisticated than previously realized [30-32]. MDR1-Pgp contributes to anti-neoplastic resistance in at least two ways: active drug extrusion and elevation of cellular apoptotic threshold [70]. Reports linking over-expression of MDR1-Pgp to adverse treatment outcome in adult AML provided the evidence necessary to implicate this MDR phenotype as an important biologic entity to design innovative therapeutic approaches and to understand its role in patients outcomes [34]. As above reported, numerous methods are currently applied to evaluate MDR1-Pgp expression in clinical specimens. Despite efforts to establish standardized methodology, development of consensus recommendations is difficult, owing to differences in assay sensitivity or specificity, the need to distinguish between normal and malignant cells, controversy as to the minimum MDR1-Pgp threshold relevant to treatment outcome. Molecular techniques such as RT-PCR provide sensitive and often quantitative measures of MDR1 gene message, but with compromised specificity owing to non neoplastic cell contamination [78]. Immunodetection methods such as flow cytometry, immune-histochemistry and drug efflux function offer the advantage of MDR1-Pgp assessment according to phenotype morphology and transport capacity [79, 80]. The majority of published investigations traditionally use for determination of MDR1-Pgp expression level in AML cells the mAb MRK-16 since it is worldwide recognized as MDR1-Pgp specific [105]. Over-expression of MDR1-Pgp is relatively frequent in adults with AML at diagnosis and especially at relapse. In several studies of AML in adults, CR rates were significantly lower among patients with over-expression of MDR-Pgp than those without. However, interpretation of clinical data may be hampered by variation in methods and antibodies used for measuring relative levels of MDR1-Pgp expression. Nonetheless, the use of a single mAb for MDR1-Pgp typing may be not fully reliable in defining the MDR phenotype of AML blasts. In agreement with the consensus recommendation published in 1996 [108] we strongly suggest the use of two or more vendor-standardized anti- MDR1-Pgp antibody reagents that recognize different epitopes localized in the external domain of MDR1-Pgp and parallel functional assay thus improving the reliability of immunological/functional detection of MDR1-Pgp status in AML cells. The multidrug resistance phenotype MDR1-Pgp -related is thought to play a role in the outcome of therapy for some human tumors; however, a consensus conclusion is difficult to reach, owing to the variable results published by different laboratories. Many factors appear to influence the detection of MDR1-Pgp in clinical specimens, including its low and heterogeneous expression; conflicting definitions of detection end points; differences in methods of sample preparation, fixation, and analysis; use of immunological reagents with variable MDR1-Pgp specificity and avidity and with different recognition epitopes; use of secondary labeled antibodies; and differences in clinical end points [108]. The combined effect of these factors is clearly important, especially among tumors with low and variable inter patient expression of MDR1-Pgp. In this context the general conclusions emerging from the workshop organized in Memphis (Tennessee, US) more than 15 years ago by several North American and European institutions to promote the standardization of approaches to MDR1-Pgp detection in clinical specimens, are still effective and reliable for present and future studies of MDR1-Pgp associated with pharmacology treatment of tumor cells [108]. Numerous anticancer drugs, including agents commonly used in the treatment of AML are MDR1-Pgp substrates or functionally related to the MDR family of proteins [109]. 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 [30-32]. 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 [86, 87]. Finally, by a further an increase of the selective pressure condition, the MDR1-Pgp may emerge as the unique and very efficient drug transporter machinery expressed on MDR cells. However, a different scenario of modulation of ABC transporters in *ex vivo* model has reported by Hu *et al.*, [110] who examined whether MDR1-Pgp expression and function in leukemic blasts is altered after a short exposure to anthracycline analogues. They found significant over-expression of functional MDR1-Pgp in AML blast cells after 16 hours with an intensity depending on cytotoxic drug used. In the same study up regulation of MDR1-Pgp expression is also observed

ex vivo in patients in which blasts were MDR1-Pgp negative at clinical presentation that convert in high MDR1-Pgp positive blasts from 1 to 5 months after the selective pressure of standard chemotherapy regimen. These data suggest that up regulation of the MDR1 gene may represent a normal response of leukemic cells to cytotoxic stress and may contribute to clinical drug resistance. Whereas most studies performed to date in AML showed an influence of MDR1-Pgp expression on remission rate, for MRP1 expression, the controversy regarding prognostic impact for survival is still a matter of debate [111, 112]. The reasons for these divergences may be due of small number of patients in some studies, missing cytogenetic data for performing valid multivariate analyses or comparison of patients who are treated according to different protocols. To this regard, clinical studies conducted by Filipits *et al.* [111] on the impact of MRP1 expression level in the outcome of AML patients indicate that MRP1 is expressed in patients with *de novo* AML but, in contrast to MDR1-Pgp, does not predict for outcome of induction chemotherapy or survival. Multiple methods have been investigated for assessing MDR in cell lines and in AML blast patients, offering the potential for accurate identification of gene over-expression or protein up regulation by semi-quantitative RT-PCR [78]. However, the combination of flow-cytometry studies conducted by specific mAbs to the external MDR1-Pgp domain and parallel controls with calibrated MDR cell lines where the number of MDR1-Pgp molecules are previously determined by appropriate flow cytometric systems remain the most reliable methodologies for MDR1-Pgp typing of AML blast cells [113]. Particular attention should be pay to specificity of mAbs in defining the MDR1-Pgp detection. In fact, some of the currently used mAbs for determining MDR1-Pgp expression are not entirely MDR1-Pgp specific with subsequent erroneous clinical interpretation of the medical and biological reasons of relapse/remission rate in AML patients. For example C219 one of the most used mAb for MDR1-Pgp typing presents cross-reactivity with 185- kDA transmembrane HER2 protein which is over-expressed in several carcinoma [114] and may cross-reacts with the heavy chain of muscle myosin in cardiac and skeletal muscle [115]. mAbs JSB1 [116] and C494 [117] often used for immunohistochemistry and biochemical studies of MDR1-Pgp present cross-reactivity with piruvate carboxylase an enzyme abundantly expressed at the mitochondrial level of the cells. Finally mAbs C219 and JSB-1 stain blood group A carbohydrate determinants which represent problem of quality control for immunohistochemical analysis [118]. In conclusion the use of the above mentioned mAbs should be precluded to determine the MDR phenotype of AML cells. In contrast a pivotal study that should be used as referenced methodological procedure for MDR1-Pgp expression in AML blast cells was conducted by Leith and co-workers in 352 newly diagnosed AML patients (median age, 44 years) registered to a single clinical trial (SWOG 8600) [119].

In this work, the expression of the MDR1-Pgp, on gated leukemic blasts compared with control cells was measured using a modification of the Kolmogorov-Smirnov (KS) that identifies even small differences in fluorescence level. This methodology used two distinct mAbs which recognize two distinct epitopes localized on the external surface of MDR1-Pgp but differ for the level of binding affinity. By the comparison of staining intensity of the mAbs MM4.17 [88] and MRK16 [105] on 352 AML blast samples it appears evident that these two mAbs possess different affinity and specificity towards the extracellular domain of MDR1-Pgp molecule. In fact, mAb MM4.17 stains 89, 37, 25, 200 samples with bright, moderate, dim and negative patterns respectively. In contrast mAb MRK16 shows significant difference in flow cytometry detection of the same blast samples with 64, 35, 24 and 228 bright, moderate, dim and negative patterns respectively. By analyzing these results it appears evident that the same AML blast sample may be differently categorized in MDR1-Pgp expression level according to the mAb used. Furthermore, we found that the referenced mAbs MRK-16 [105], UIC2 [120] and MC57 [121] are not appropriate reagents for MDR typing of AML cells since they tend to under evaluate the percentage of MDR1-Pgp positive cells which are intercepted by mAb MM4.17 [88, 122, 123]. Furthermore, the MDR1-Pgp typing of AML patients with the mAb 4E3 showing the lowest affinity among all mAbs to MDR1-Pgp so far tested [124] may give erroneous evaluation of MDR1-Pgp expression and misleading indications in clinical setting. Furthermore, the utilization of MDR1-Pgp dye-substrates for monitoring functional activity of MDR1-Pgp by efflux assay should corroborate [125] not substituting one of the most accurate methodology for MDR typing. Taking in aggregate all these findings in MDR1-Pgp expression on AML cells it cannot ruled out that some of previously reported data on the MDR1-Pgp expression on AML are erroneously interpreted and should be critically reviewed. For our study of MDR1-Pgp expression on AML cells and other hematopoietic tumors we used high specific and affinity mAbs MM4.17 [88], MM6.15 [126] and MM12.10 [127] originated by innovative somatic cell genetics strategy for the isolation of mAbs designed to intercept even very low level of MDR1-Pgp expression in tumor samples [123]. Our elevated confidence in these mAbs it is based on a series of studies conducted in several independent laboratories. In this context, Kuo and co-workers [128] by analyzing of MDR1-Pgp expression in normal and malignant endometrium by RT-PCR and immunohistochemistry adopting for comparison Kruskal-Wallis and Bonferroni tests, setting the P value at 0.05 found a correlation of 80% between molecular and immunechemical investigation with mAb MM4.17. In the

same study, control MDR cells expressing low level of MDR1-Pgp, were stained by mAb MM4.17 and not by mAb MRK16. This result indicates that the use of mAb MRK-16 as unique immunoreagent for MDR1-Pgp detection even tough world-wide and traditionally adopted for MDR typing may give erroneous MDR1-Pgp evaluation of AML samples.

CONCLUSION

Advances in genomics technologies have identified AML as a genetically heterogeneous disease, and many patients can now be categorized into clinical-pathologic subgroups on the basis of their underlying molecular genetic defects. It is hoped that enhanced specificity of diagnostic classification will result in more effective application of targeted agents and the ability to create individualized treatment strategies. To this regard may be of high medical relevance the monitoring of the MDR1-Pgp expression in blasts cells in the bone marrow during chemotherapy regimen to eventually identify those patients who will need additional treatment. Significant efforts toward improving the clinical outcome of elderly and pediatric AML patients are still needed. A contributing factor to the relatively slow progress may be that, despite significant heterogeneity in AML cells, the clinicians have historically treated AML uniformly. The idea of identifying sub-populations with high reliable MDR1-Pgp expression and function within AML for treatment stratification is likely to play an increasingly important role in future therapeutic strategies. In this context we consider that reliable expression level of MDR1-Pgp should be included in the initial work-up of patients submitted to AML immunochemotherapy and MDR1-Pgp should be taken into consideration in risk classification and clinical setting.

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Conflict of interest statement

There are no potential conflicts of interest or any financial or personal relationships with other people or organizations that could inappropriately bias conduct and findings of this study.

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