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Targeting MDR1-P-glycoprotein (MDR1-Pgp) in immunochemotherapy of acute myeloid leukemia (AML)

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Abstract

Background. Monoclonal antibodies represent the fastest growing sector of pharmaceutical biotechnology and a number of antibody-based biopharmaceuticals have been approved for cancer treatment. However, in many cases the antibodies used for the treatment of tumors offer only a modest survival benefit to cancer patients.

Aims. In the present review-article we intend to analyze: i) the curative regimen gemtuzumab ozogamicin (GO) -mediate characterized by the absence of cytotoxic drugs MDR1-Pgp substrates to overcome the mechanism of action of this multidrug transporter, ii) the safety and efficacy of MDR reversing strategy in AML outcome and, iii) chemical and biological MDR modulators playing a dual relevant medical role as a therapeutic and MDR reversing agents but not yet entered in the clinical setting of AML. Since the similar multidrug transporter protein MDR1-Pgp and its down modulation factors may affect safety and efficacy of already generated antibody drug conjugates (ADCs) a comprehensive overview of the most clinically representative immunoconjugates is reported.

Discussion. ADCs represent one of the most promising strategies to enhance the antitumor activity of antibodies. ADCs comprise an antibody (or an antibody fragment) conjugated to a cytotoxic drug via a chemical linker. The therapeutic concept of ADCs is to use an antibody as a vehicle to selectively delivering a cytotoxic drug specifically to a tumor cell, in most cases by means of binding to target cell surface antigen. As a consequence, ADCs have significant potential for enhancing the antitumor activity of "naked" antibodies and reducing the systemic toxicity of the conjugated drugs.

Key words
- acute myeloid leukemia (AML)
- immunoconiugate
- gemtuzumab ozogamicin (GO)
- MDR1-P-glycoprotein
- multidrug resistance (MDR)
- MDR reversing agents

INTRODUCTION

The rationale for the development of antibody-drug conjugates (ADCs) is to combine the specificity of targeting inherent to monoclonal antibodies with the ability to deliver highly toxic chemotherapeutic agents that cannot be administered systemically [1]. The drugs used in ADCs currently in the clinic or preclinical testing can be divided into two categories, those targeting DNA (calicheamicin) and those targeting microtubules (maytansine, auristatin) [2]. Calicheamicin-gamma1 is a highly cytotoxic enediyne antibiotic that binds to the minor groove of DNA and induces double-strand DNA breaks that result in cell death [3, 4]. However, resistance to anticancer compounds with the high cytotoxic potency delivered by antibody conjugate remains a challenging issue for patients and their physicians. In order to contribute in understanding the success and failure of pharmacological strategies designed to improve the efficacy of ADCs by down modulating MDR1-Pgp, we intend to dissect some of the biological factors playing a critical a role in the clinical safety and efficacy of gemtuzumab ozogamicin (GO) [5]. The impressive number of clinical experimentations and molecular-genetics studies conducted for re-assessing AML treatment [6] is a collection of data which could be utilized for modeling the curative regimen of other ADCs as well as to design innovative immunotherapeutic strategies. In particular, in the present review-article we intend to analyze the curative regimen GO -mediate characterized by the absence of cytotoxic drugs MDR1-Pgp substrates to overcome the mechanism of action of this multidrug transporter. Furthermore, chemical and biological agents playing a dual role as a therapeutics and multidrug resistance (MDR) reversing agents will be examined for their medical relevance in acute myeloid leukemia (AML). Since similar multidrug transporter protein and its down modulation factors may affect safety and efficacy of newly generated ADCs a comprehensive overview of

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selected immunoconjugates targeting microtubules *(Figure 1)* and DNA *(Figure 2)* is herein reported.

ANTIBODY DRUG CONJUGATE: THE NEW FRONTIERS OF CANCER THERAPY

Monoclonal antibodies represent the fastest growing sector of pharmaceutical biotechnology and a number of antibody-based biopharmaceuticals have been approved for cancer treatment [7]. However, in many cases the antibodies used for the treatment of tumors offer only a modest survival benefit to cancer patients. ADCs represent one of the most promising strategies to enhance the antitumor activity of antibodies. ADCs comprise an antibody (or an antibody fragment) conjugated to a cytotoxic drug via a chemical linker [8]. The therapeutic concept of ADCs is to use an antibody as a vehicle to selectively delivering a cytotoxic drug to a tumor cell in most cases by means of binding to a target cell surface antigen [9]. As a consequence, ADCs have significant potential for enhancing the antitumor activity of "naked" antibodies and reducing the systemic toxicity of the conjugated drugs. Pre-clinical studies have clearly shown that incorporation of highly potent drugs (free drug potency in the order of 10-9 to 10-11 M) to therapeutic anticancer antibodies results in more effective reagents than using low potency drugs already approved for cancer therapy such as doxorubicin (free drug potency around 10-7 M) [8]. Auristatins and maytansinoids, are highly active inhibitors of microtubule assembly/function [9].

Brentuximab vedotin, SGN-35 (SGN-35, Seattle Genetics) [10] is generated by conjugating SGN-30, a chimeric anti-CD30 monoclonal antibody to the synthetic antitubulin agent monomethyl auristatin E (MMAE), an analogue of the marine natural product dolastatin, through an enzyme-cleavable valine-citrulline dipeptide. Brentuximab vedotin binds to the extracellular domain of CD30, becomes internalized by clathrin-mediated endocytosis, and subsequently travels to the lysosome where proteases cleave the linker peptide and release MMAE into the cytosol. MMAE binds to tubulin and potently inhibits microtubule polymerization, inducing G_2-M phase growth arrest and apoptosis in CD30-expressing lymphoma cells *(Figure 1)*. *In vitro*, the drug is found to be potent and selective against CD30-positive tumor-cell lines, and activity is observed in models of Hodgkin's lymphoma (NHL) and anaplastic large cell lymphoma (ALCL) in mice with severe combined immunodeficiency. On August 19, 2011 Seattle Genetics announced that the US FDA accepted two Biologics License Applications (BLAs) for Brentuximab vedotin, one for the treatment of relapsed or refractory ALCL patients, and the other for treatment of patients with NHL, in which the drug also shows clinical benefit. Trastuzumab emtansine [11] is another ADC, which contains a maytansine derivative (DM1) conjugated to the FDA-approved trastuzumab, a humanized IgG1 antibody specific for the human epidermal growth factor receptor 2 (HER2/neu). Clinically, T-DM1 has a consistent pharmacokinetics profile and minimal systemic exposure to free DM1, with no evidence of DM1 accumulation following repeated T-DM1 doses. Although a few covariates are shown to affect inter individual variability in T-DM1exposure and clearance in population-pharmacokinetics analyses, the magnitude of their effect on T-DM1 exposure is not clinically relevant. Phase I and phase II clinical trials of T-DM1 as a single agent and in combination with paclitaxel, docetaxel, and pertuzumab show clinical activity and a favorable safety profile in patients with HER2-positive metastatic breast cancer [12]. The lower general toxicity and its associated increase in life quality and better physical appearance in a patient population that are mainly females are important improvements of T-DM1 that certainly deserve attention.

Very recently, on February 23, 2013 Roche announced that the US FDA approved T-DM1 for the treatment of people with HER2 positive metastatic breast cancer who have received prior treatment with Herceptin (trastuzumab) and a taxane chemotherapy. T-DM1 is being approved with a black box warning that the drug can cause liver toxicity, heart toxicity, and death. GO (Mylotarg) is another ADC that was market in US since its FDA accelerated approval in May 2000 [5]. This procedure requires that additional clinical trials be completed after approval with due diligence to verify and describe the clinical benefit. GO is a humanized IgG4 anti CD33 antibody conjugated to calicheamicin-gamma1, a highly potent antibiotic that induces apoptosis in cancer cells by a DNA binding mechanism [3, 4]. Calicheamicin-gamma1 works at very low concentrations, allowing its use at low doses *in vivo* [13]. *In vitro*, GO was 2000-fold more potent than the parental drug that preceded its development and was the first ADC approved by the US FDA to treat recurrent AML in patients aged 60 and older who were not candidates for standard chemotherapy. GO is a heterogeneous formulation, containing approximately a 1:1 mixture of conjugated (one to eight calicheamicin moieties per IgG4 molecule, with an average of 4-6 moieties randomly linked to solvent exposed lysyl residues of the antibody) and unconjugated antibody [5, 13]. The myeloid cell surface antigen CD33 represents an attractive target, as it is expressed from about 90% of AML patients [14]. To confirm clinical benefit, Wyeth Pharmaceuticals (Philadelphia, PA), the commercial sponsor of the GO marketing application at that time, committed to conduct a randomized trial to determine if the addition of GO to daunorubicin and cytarabine would improve the OS of patients with *de novo* CD33 AML. In spite of auspicious preclinical and initial clinical results, follow-up studies showed no additional benefits to AML patients, and an increased fatality rate in chemotherapy plus GO-treated patients when compared to standard treatments (5.7% *vs* 1.4%) [6].

For these reasons, the product was voluntarily withdrawn by Pfizer from the US market in June 2010. Subsequent findings in four additional randomized trials comparing standard induction chemotherapy with and without GO in newly diagnosed AML pa-

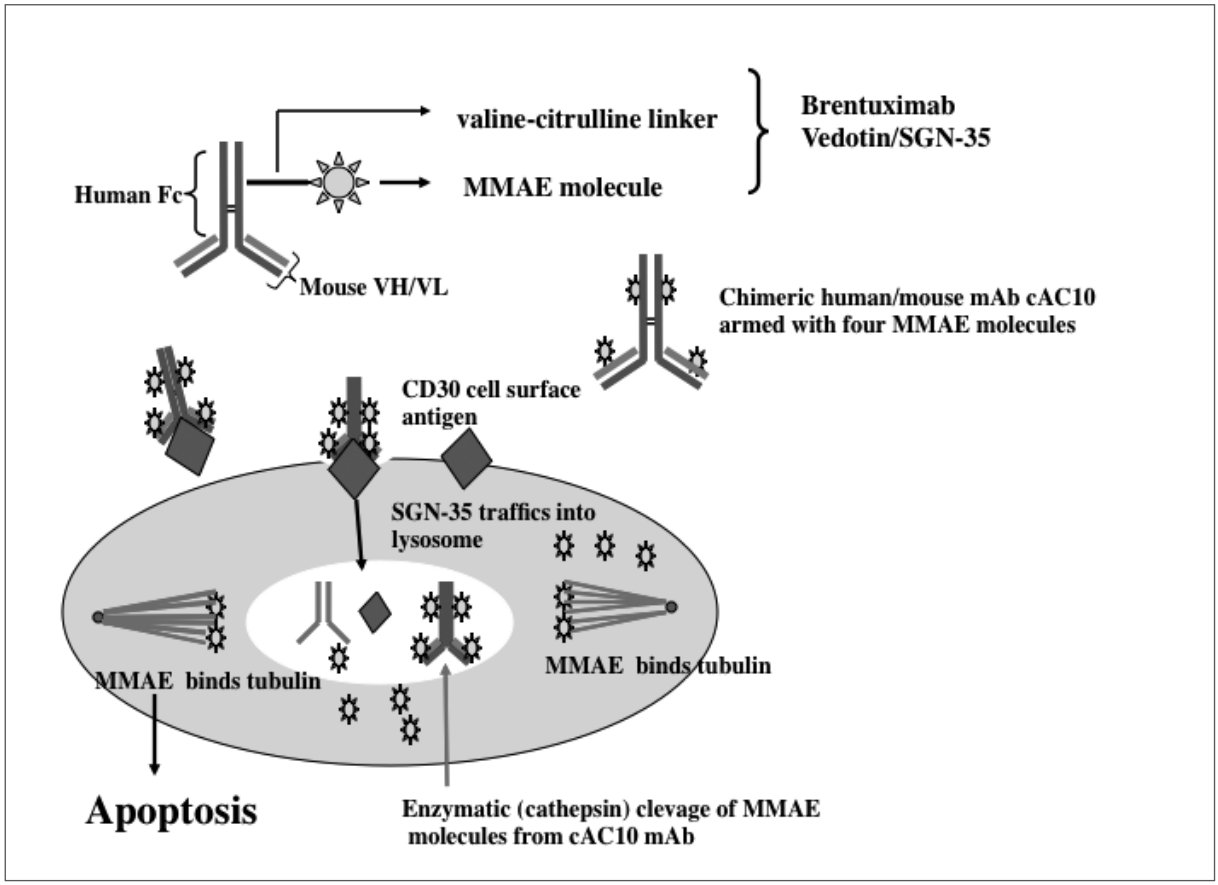

Figure 1
Trafficking and activation of the antibody drug conjugate (ADC) of brentuximab vedotin
The antibody-drug conjugate (ADC) brentuximab vedotin (Seattle Genetics) was developed to increase antitumor activity by linking a potent antimicrotubule agent, MMAE, to the chimeric CD30 monoclonal antibody cAC10 via a protease-cleavable linker. This ADC binds to CD30, is rapidly internalized in the cell, and then traffics to the lysosomal compartment where the dipeptide linker is cleaved by cathepsin. Once released, binding of MMAE to tubulin disrupts the microtubule network within the cell, induces cell-cycle arrest, and results in apoptotic death of the tumor cell

tients [15-18] stood in contrast to the phase III confirmatory study and suggested clinical benefit among certain patients – those whose AML is characterized by either "good" or "intermediate" cytogenetics risk. Inotuzumab ozogamicin is an ADC composed of the humanized mAb G544 of IgG4 isotype that specifically recognizes human CD22, and the derivative of calicheamicin. The majority (> 90%) of NHLs are of B-cell origin, with CD22 being expressed 60% to > 90% of B-lymphoid malignancies, CD22 has many of the ideal properties for an ADC target. Unconjugated G544, having no effector function, has no antitumor activity; instead, conjugation with the cytotoxic payload confers potent dose-dependent cytotoxicity in *in vitro* and *in vivo* animal tumor models [19]. Inotuzumab ozogamicin displayed greater single-agent therapeutic benefit than either CVP (cyclophosphamide, vincristine, and prednisone) or CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) in xenograft models, and it induced superior antitumor activity when co-administered with standard chemotherapeutic regimens [20]. Nevertheless, as with GO, inotuzumab ozogamicin is not effective in MDR1-Pgp-positive sublines (Daudi/MDR and Raji/MDR cells), and MDR modifiers restored the cytotoxic effect [21]. In clinical samples, the cytotoxic effect is inversely related to the amount of MDR1-Pgp and to intracellular rhodamine-123 accumulation. Conversely, the effect positively correlated with the amount of CD22 [22]. However, resistance to drugs with high cytotoxic potency carried out within tumor cells by the specific antibody remains a challenging issue for patients and their physicians. In fact, all drugs forming the above mentioned ADCs (calicheamicin, monomethyl auristatin E and maytansine derivatives) are MDR1-Pgp substrates [23, 24] and the presence of this entity on tumor cells may attenuate or completely abrogate their curative potential. In this context the above mentioned ADCs are designed for treatment of tumors from which MDR1-Pgp is constitutively expressed (AML) or emerge as MDR variants after the selection of chemotherapy treatment (breast cancer and NHL). Reports linking overexpression of the MDR1-Pgp to

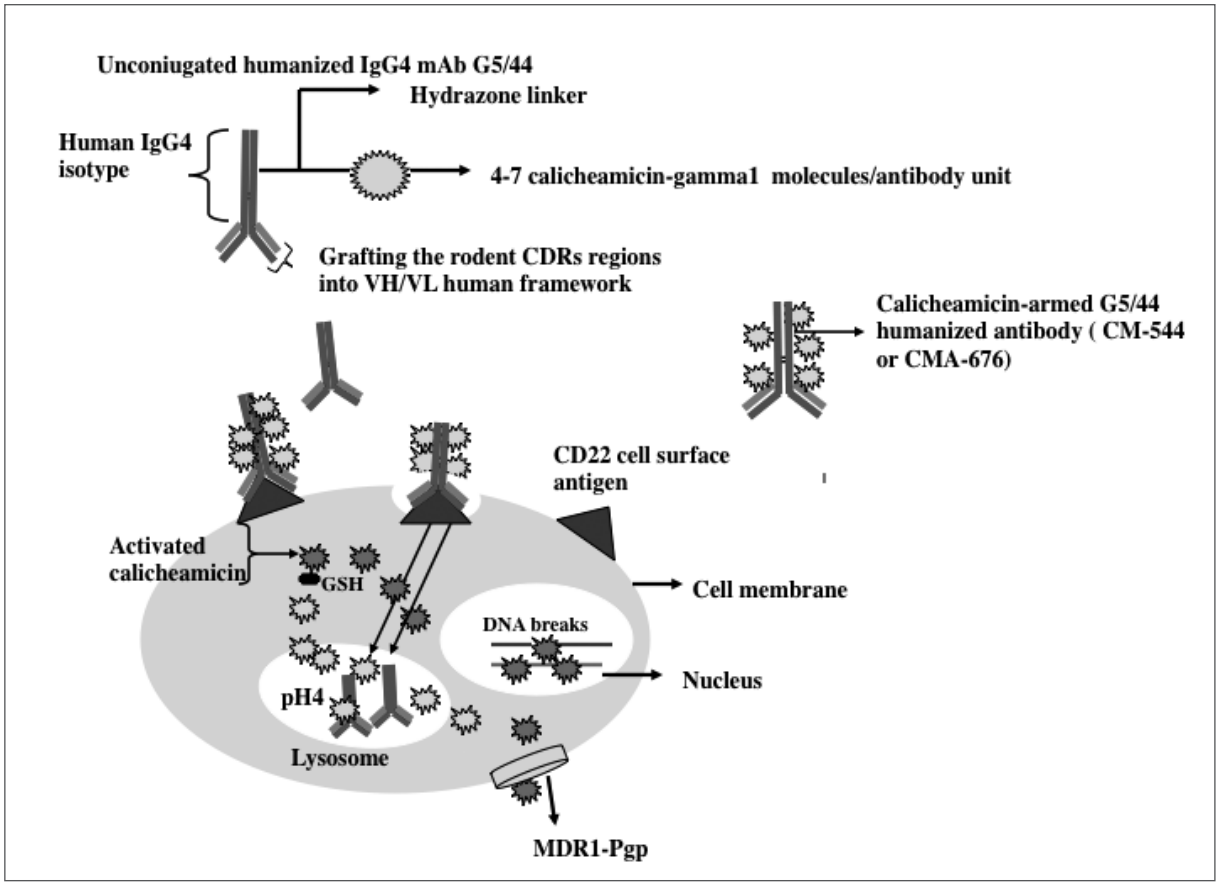

Figure 2
Trafficking and activation of the antibody drug conjugate (ADC) Inotuzumab Ozogamicin
CD22 is expressed on the surface of mature B lymphocytes and their malignant counterparts but not on other non-B lineages including hematopoietic stem cells. CD22 is rapidly internalized on binding to anti CD22 mAb. These properties make CD22 a suitable molecular target for antibody-targeted calicheamicin therapy for B-lymphoid malignancies. The humanized anti-CD22 mAb (G5/44) was conjugated to calicheamicin by, an acid-labile AcBut hydrazone linker a designed as CMC-544 immunoconjugate. After binding and internalization, CMC-544 traffics to lysosome, where the acid-labile AcBut hydrazone linker is cleaved in the acid environment of lysosome. Hence calicheamicin derivative is released intracellularly. The reduction to the active enediyne form requires glutathione. The active enediyne form (calicheamicin-gamma1) binds to the minor groove in DNA and causes double-strand breaks, resulting in cell death. MDR1-Pgp mediated efflux may be a mechanism of drug resistance in several type of leukemic cells

adverse treatment outcome in adult AML, NHL and breast cancer provided the evidence necessary to implicate this MDR phenotype as an important biologic target for pharmacological modulation.

MDR1-Pgp IN AML CELLS

MDR1-Pgp (ABCB1) as well as the family of structurally and functionally related proteins, are plasma membrane transporters which are able to efflux out of the cell a variety of substrates including chemotherapeutic agents [25]. Such proteins which include the multi-drug resistance-associated protein (ABCC1, MRP1) [26] and the breast cancer resistance protein (BCRP/ABCG2) [27] like MDR1-Pgp, lowers intracellular drug accumulation by promoting drug efflux and MDR. Although the expression of MDR1-Pgp plays an important role in the MDR phenotype of AML, there are discrepancies in studies performed to evaluate the importance of MRP1 and ABCG2 in this cancer [28, 29]. The discovery of MDR1-Pgp made available a potential target for pharmacologic down-regulation of efflux-mediated chemotherapy resistance [30]. In AML patients, a neoplasm characterized by proliferation of poorly differentiated myeloid progenitor cells, leukemic cells often express MDR1-Pgp at high levels, which may lead to the development of resistance to chemotherapy [31]. In this context, the expression of MDR1-Pgp the most studied member of ABC family of transmembrane proteins is the main factor responsible for multidrug resistance in AML. Expression of MDR1-Pgp correlates with a reduced complete remission (CR) rate and shorter durations of overall survival (OS) or disease-free survival (DFS) [32]. Thus, AML seemed to be a cancer for which the addition of drug efflux inhibitors to the chemotherapeutic regimen would improve outcomes of patients. Since the clinical relevance of MDR1-Pgp in the pharmacology of calicheamicin-gamma1 we focus our attention on AML curative regimen containing GO and cytotoxic drugs non MDR1-Pgp substrates.

Table 1
Selected AML clinical studies combining gentuzumab ozogamicin (GO) with drugs non MDR1-Pgp drug substrate

Pharmacological treatment	AML patients	Outcomes	Remarks and references
Cytarabine + GO: (3 mg/m2)	16 patients elderly patients (64-82 years) with newly diagnosed AML	*Favorable*, intermediate I cytogenetic group, 11 (91.7%) achieved CR. None achieved CR with intermediate II adverse cytogenetic profile	The median disease-free survial and OS was 10.9 and 18.8 months for CR patients [44]
Cytarabine and G-CSF (G-AraMy 1 and G-AraMy 1) + GO: 6 mg/m2 i.v. From 6 mg/m2 to 3 mg/m2 in maintenance therapy	53 elderly patients [median age 69 years (range 65-77)] with untreated/primary refractory/relapsed AML	After induction treatment, 23 patients (43%) achieved CR and one patient (2%) CRp. Eleven patients (21%) had PR, 11 patients (21%) resulted refractory to treatment, and seven (13%) patients died	After consolidation CR was 57% (30 of 53 patients). Median DFS was 8 months (range 2-23+). Median OS 9 months (range 2–24+), with a 12-month OS rate of 28% [46]
High dose cytarabine 3 g/m2 over 3 hours daily for 5 days (HiDAC) + GO 9 mg/m2. CALGB study 19902	37 patients with relapsed or refractory AML	12/37 (32%) patients with relapsed AML achieved CR. Median OS was 8.9 months	No grade 4 hepatic VOD was observed [33]
Fludarabine and cytarabine (BIDFA) + GO 3 mg/m2. CML-BP patients were treated with tyrosine kinase inhibitors	107 patients with refractory/relapsed AML, intermediate and high-risk MDS, and CML-BP	27 (26%) patients responded with a CR rate of 21% and CRp of 5%. The CR 12 months, less and relapsed were 56%, 26%, and 11%, respectively	BIDFA is safe with a low 4-week mortality rate of 9% [51]

AML: acute myeloid leukemia; GO: gemtuzumab ozogamicin; CML-BP: chronic myeloid leukemia in myeloid blast phase; MDS: myelodysplastic syndromes; VOD: veno-occlusive disease; CR: complete remission; CRp: complete remission with incomplete platelet recovery; OS: overall survival; RFS: relapse-free survival; PR: partial response.

GEMTUZUMAB OZOGAMICIN TREATMENT COMBINED WITH CYTOTOXIC DRUGS NON MDR1-PGP SUBSTRATES

Outcomes for patients with AML, particularly those over age 60 years, have not significantly improved in the past 30 years and conventional cytarabine and anthracycline-based chemotherapy remains the most effective pharmacological treatment [33, 34]. Although little change is achieved in chemotherapy regimen, supportive pharmacologic care (including antiemetics, and antibiotics) has improved significantly and has made this chemotherapy more tolerable [35]. The rate of relapse, however, remains high, and the overall outcome in older adult populations is poor. Conventional chemotherapy regimens induce CR in 65% to 85% of patients younger than 60 years of age. Of those achieving a CR, only 30% to 40% can expect DFS. In older adults (60 years of age), results are even more dismal, with CR achievable in 40% to 55% of patients. Of those who do achieve a CR, only 10% to 20% are still alive 3 years out from diagnosis [35]. This low chance of durable remission comes at a price of high treatment-related mortality (20% or higher compared to less than 10% in the younger adult population). A number of studies have explored more intensive up-front chemotherapy, addition of cytotoxic drugs, and even extended maintenance therapy, without demonstrable improvement in survival outcomes

Table 2
Selected phase II clinical studies combining GO with drugs non MDR1-Pgp substrates and CsA as MDR reversing agent

Pharmacological treatment	AML patients	Outcomes	Remarks and references
GO 6 mg/m2 iv on day 1; fludarabine 15 mg/2 and cytarabine 500 mg/m2 twice daily on day 2-6; (BIFDA) CsA 6 mg/kg loading dose before GO, followed by 16 mg/kg continuous iv infusion on days 1 and 2	60 patients, median age 57 years (27-76) with 66% AML, 34% MDS. Patients show intermediate-risk and adverse cytogenetics profile. Diseases status 66% AML untreated	CR, 46%; CRp, 2%; MS, 8 months; 1 year LFS survival 27%. Response rate AML and MDS were similar	Infections 38%; grade 3-4 hyperlirubinaemia 31%; elevation hepatic enzymes 7%; VOD 7% [52]
GO 6 mg/m2 iv on day 1; BIFDA; CsA 6 mg/kg loading dose before GO, followed by 16 mg/kg continuous iv infusion CsA on days 1 and 2	32 patients, median age 53, years > 18 (range 65-77) with untreated/primary refractory/ relapsed AML. Disease status, first relapse or primary refractoty	CR 28%; CRp 6% MS 5-3 months	Hepatotoxicity, Grade 3-4 hyperlirubinaemia 44%. Grade 3-4 elevation of hepatic enzymes 18%. 9% patients developed VOD and died [53]

AML: acute myeloid leukemia; GO: gemtuzumab ozogamicin; CsA: Cyclosporin A; MDS: myelodysplastic syndromes; VOD: veno-occlusive disease; CR, complete remission; CRp: complete remission with incomplete platelet recovery; OS: overall survival; LFS: leukemia-free survival.

[33-35]. Thus, for almost all older AML patients, therapy for relapsed disease will be a consideration. To face this dramatic medical need, antibody-tailored therapy consisting of the anti CD33 calicheamicin-gamma1 armed antibody is a promising new curative approach introduced for the treatment of AML patients [5]. The purpose of this therapy is to deliver in AML cells calicheamicin-gamma1 which is one of the most cytotoxic agent so far isolated, thereby diminishing the toxic side effects and probably the treatment related morbidity and mortality. The cell surface antigen CD33, is a 67 kDa glycoprotein, which is normally expressed during myeloid differentiation, is expressed in 90% of leukemic blasts and in CD34+/CD38-/CD123+ AML stem cells [36]. Binding of GO to the CD33 antigen leads to internalization of the drug-antigen complex and hydrolytic release of the toxic calicheamicin-gamma1 components. After internalization, calicheamicin-gammma1 is released from the lysosomes. It subsequently enters the nucleus where it cleaves double-stranded DNA and induces cell death (*Figure 2*). However, we and others have recently demonstrated that the anti tumor antibiotic calicheamicin-gamma1 may be included in the large family of anti cancer drugs MDR1-Pgp substrate [28, 37]. Hence, drug efflux mediated by MDR1-Pgp should result in resistance to GO and predicts for adverse outcome [32]. This finding based on several in *in vitro/in vivo* studies has been recently confirmed by mathematical model predicting that patients who have low MDR activity and a high CD33 production rates are most likely to benefit from GO [38]. These two parameters can be easily evaluated by flow cytometry, after *in vitro* exposure of blast cells to specific mAbs. These findings further suggest that GO efficacy could be enhanced when used after the leukemic tumor burden was modestly lowered, *e.g.* by alternative cytoreductive agents [38, 39]. Several independent *in vitro* studies demonstrated that inhibition of MDR1-Pgp function using MDR reversing agents effectively increases GO cytotoxicity even though the severe toxicity observed during clinical trials fails to translate this MDR reversing strategy as consolidated medical option [40-42]. These observations underline the biological and clinical relevance in designing curative AML strategies to circumvent MDR1-Pgp mediated drug resistance by using a combination of drugs that are not recognized by this pathway. This therapeutic approach may offer, in principle, the advantages of pharmacological treatment characterized by the presence of calicheamicin-gamma1 as unique MDR1-Pgp substrate thus lowering the biological elements involved in the efflux mechanism of MDR1-Pgp expressed on the cell surface of AML cells and increasing the possibility to identify an effective and safety MDR down modulation strategy to overcome MDR1-Pgp mediated drug resistance. In *Table 1*, we report selected GO curative regimens that combine the AML treatment with agents non MDR1-Pgp substrates. The elimination of cytotoxic drugs non MDR1-Pgp substrates in induction/consolidation therapy of AML was originally suggested by Goemans and co-workers [43]. They studied cross-resistance between calicheamicin-gamma1 with drugs that are currently used in the majority of AML therapies. The results showed that there is marked cross-resistance between calicheamicin, the

anthracyclines idarubicin (Spearman's = 0.73, P < 0.0001, n = 23), daunorubicin (p = 0.61, P < 0.0001, n = 103) and the anthracenedione mitoxantrone (p = 0.52, P = 0.039, n = 16). In addition, there is moderate cross-resistance with etoposide (p = 0.42, P < 0.0001, n = 101). No cross-resistance was observed between calicheamicin-gamma1 and cytarabine (p = 011), 6-thioguanine (P = 020) or L-aparaginase (P = 021). The adverse effects of functional MDR1-Pgp expressed on the cell surface of AML CD33 may be dual: calicheamicin-gamma1 and cross resistant drugs may be intercepted by MDR1-Pgp molecules and efflux out lowering their concentration under an effective curative level. Further sub-lethal concentrations of cytotoxic drug may induce activation of ABCB1 gene amplification thus increasing the level of the MDR1-Pgp product and resistance of AML to chemotherapy. The most obvious considerations of these adverse effects should be the elimination of cytotoxic drugs showing cross resistance with calicheamicin-gamma1 and combining AML therapy with MDR reversing agents compatible with tolerable pharmacokinetics profile of the administered drugs. The combination of GO with cytarabine and/or L-asparaginase that are compounds non MDR1-Pgp substrates should be considered as an alternative strategy to circumvent drug resistance mediated by this pathway. To this regard, high response rate for treatment with GO and cytarabine was achieved by Tavor *et al*., [44] using the curative potential of low dosage of GO (3mg/m2) which was found to be very effective and safe by the pivotal study of Taksin *et al*., [45]. However, this was true only in patients in the *favorable* or *intermediate*-I cytogenetic risk groups. Of the 12 patients with AML in the *favorable* and *intermediate*-I cytogenetic groups, 11 (91.7%) achieved CR. By comparison, of all 4 patients in the *intermediate*-II or *unfavorable* genetic groups, none of the patients achieved CR (P = 0.003). The median disease-free survival and OS was 10.9 and 18.8 months, respectively, for patients who achieved CR. The estimated median survival was 15 months in the *favorable* and *intermediate*-I cytogenetic risk groups and only 4.4 months in the *intermediate*-II and *unfavorable* risk groups (P = 0.008). By adding G-CSF to the GO combined with cytarabine, Fianchi *et al*., [46] evaluated the safety and efficacy in this regimen (G-AraMy) in elderly patients with poor-prognosis AML. The authors found that G-AraMy could be a useful treatment approach, with acceptable toxicity. The MDR reversing activity attributable to G-CSF [47] may play a key role in the excellent results (CR/CRp: 23/1, median OS = 9 months) obtained by G-AraMy regimen. This therapeutic option may be complemented and extended to other drugs effective in AML therapy but non MDR1-Pgp substrates such as fludarabine [48]. This consideration provides a rationale for more extensive and more intensive testing of combinations of cytarabine and/or L-asparaginase or cytarabine and fludarabine. In relapsed/resistant and in secondary AML, increasing the dose of cytarabine and combining cytarabine with fludarabine might be more rewarding than administering anthracyclines or other MDR1-Pgp drug substrates. The FLAG regimen has featured in several studies as induction therapy for relapsed AML and for patients who failed to achieve remission with standard daunorubicin and cytararabine regimens and has been a successful strategy in refractory AML with documented MDR1-Pgp inducing multidrug resistance [49]. Furthermore AML cells with the highest expression of MDR1-Pgp has the greatest differential response to FLAG. A group of institutions, which used FLAG for remission induction in *de novo* AML, published a case control study of MDR1-Pgp and induction regimens with and without fludarabine, which suggested that this agent was of benefit in MDR1-Pgp-positive cases [50]. Recently, this trend in the elimination of MDR1-Pgp substrates to design new and more effective therapy for AML was clinically experimented by Jabbour *et al.,* [51]. They evaluate the efficacy and safety of the combination of twice-daily fludarabine and cytarabine (BIDFA) in patients with refractory/relapsed AML, high-risk myelodysplastic syndromes (MDS), and chronic myeloid leukemia in myeloid blast phase (CML-BP). In this study the concentration of GO was harmonized with most recent findings [15-18] claiming the safety and efficacy of low concentration (3mg/m2) of the immunoconjugate. Patients with CML-BP were allowed to receive concomitant tyrosine kinase inhibitors. The outcome of this novel curative regimen were encouraging with an overall CR of 26% in a heavily pretreated population. Nevertheless, two clinical phase II studies conducted by Tsimberidou *et al*., [52] on untreated/relapsed AML combining twice daily fludarabine and cytarabine with GO and the MDR reversing agent CsA (*Table 2*) show severe toxicity and modest leukemia free survival period. Very likely, drug concentrations and administration schedules used in these investigations are in contrast with the optimal drug/reversing agent combination (BIFDA + CsA) suggesting that multivariate therapeutic as well as phenotypic and genotypic factors may affect AML outcomes [52, 53].

MDR REVERSING STRATEGIES IN AML TREATMENT

The identification of MDR1-Pgp as one of the most important drug efflux system by which AML cells attenuate the citotoxic potential of GO has prompted many efforts to identify MDR reversing agents that by inhibiting MDR1-Pgp may improve or restore in a medical significant level the susceptibility of AML to calicheamicin-gamma1. In adult AML patients, the overexpression of MDR1-Pgp is associated with reduced cellular accumulation calicheamicin-gamma1 and cross resistant drugs that can be overcome by concurrent exposure to competitive MDR1- Pgp antagonists such as CsA. Southwest Oncology Group trial SWOG-9126 shows that adding CsA to a chemotherapy regimen containing infusional daunorubicin significantly reduces induction resistance in patients with high-risk AML and prolongs the duration of re-

mission and survival, confirming the role of MDR1-Pgp as an important cellular mechanism of AML resistance [54]. The nature of this inhibition appeared to reduce the interaction of anticancer drugs with MDR1-Pgp [55, 56]. This is presumed to be via competition for transport since the MDR1-Pgp expressing cells also displayed lower CsA accumulation [57]. CsA binds to an identical site to vinblastine [55], but this site is distinct and allosterically linked to that for azidopine interaction [56]. These data provided the first evidence for multiple sites of drug interaction on MDR1-Pgp. The detailed *in vitro*, and pre-clinical, observations enabled progression of CsA to phase I clinical trials [58, 59]. However, according with Pallis and Russel [60], the anti MDR1-Pgp function exerted by CsA may also originate by its indirect inhibitory activity against the ceramide-metabolising enzyme glucosylceramide synthase (GCS), which metabolizes ceramide to glucosylceramide. High levels of GCS activity and sphingomyelin synthase activity are associated with low intracellular ceramide levels and with a drug-resistant phenotype in AML [61]. Inhibition of GCS enhances intracellular levels of the pro-apoptotic mediator ceramide, which has a key role as a mediator of daunorubicin-induced apoptosis. First generation of MDR1-Pgp inhibitors in addition to CsA include verapamil, quinine, quinidine [30]. These drugs already approved for other medical purposes are very potent and effective in *in vitro* systems by disabling MDR1-Pgp function in AML blasts and improving GO cytotoxicity [30, 31, 62]. In contrast, these agents are toxic *in vivo* if dosage is calibrated with that required for down modulating MDR1-Pgp function in MDR cell lines *in vitro*. Very likely, the MDR reversing agents are used for down modulation studies at concentrations higher than those medically acceptable. Despite these safety concerns, a randomized phase III clinical trial showed the benefit of addition of CsA to treatment with cytarabine and daunorubicin in patients with poor-risk AML [54]. Similarly, quinine was shown to increase the complete remission rate as well as survival in MDR-Pgp positive MDS cases treated with intensive chemotherapy [63], suggesting that successful MDR1-Pgp modulation is feasible. However, several other trials failed to confirm significant clinical improvement and toxic side effects were common. In this context, pilots studies incorporating the MDR1-Pgp inhibitor CsA in GO containing regimens as induction, salvage or post-remission therapy in AML does not appear to increase rates of response and survival, and an increased incidence of veno-occlusive disease (VOD) was noted in patients with high tumor loads [52, 53]. However, the phenotypic and genetic complexity of AML cellular system may affect the efficacy of therapeutic treatment; selected inhibitors administered with more appropriated schedule *i.e.*, lower and fractionated doses distributed in more days and/or in combination with biological compounds such as specific mAbs may be a more appropriated strategy for disabling the biological function of MDR1-Pgp [64]. Nonetheless, first generation of MDR1-Pgp inhibitors provided several important pieces of information: (*i*) proof of principle that MDR1-Pgp could be inhibited *in vitro*, (*ii*) MDR1-Pgp is poly-specific but with a defined selectivity (*iii*) low affinity MDR1-Pgp inhibitors does not possess clinical potential and, (*iv*) the partial benefits observed in clinical trials by combination of chemotherapeutics and MDR reversing agents provided the impetus to produce more effective compounds which now is rich of three categories of chemical modulators. To this regard, PSC-833 (valspodar), a non immunosuppressive and non nephrotoxic analog of CsA, included in the second and more biochemically sophisticated category of MDR reversing agents shows an impressive capability in *in vitro* system to disable drug transport function of MDR1-Pgp expressing AML cells [65]. However, clinical trials which incorporate one of the most potent MDR1-Pgp modulator were disappointing. Phase III trials comparing induction chemotherapy with or without PSC-833 in older patients with relapsed/refractory and chemotherapy-naïve AML were negative [66-68], despite significant correlations between outcomes and MDR1-Pgp expression and function [69]. More recently, CALGB 19808 phase III trial was designed to test the hypothesis that patients younger than age 60 years would benefit more for than older patients from MDR1-Pgp blockade despite having a lower incidence of MDR1-Pgp expression [70]. In CALGB 19808 study, 302 patients were randomly assigned to receive induction chemotherapy regimens consisting of cytarabine (A), daunorubicin (D), and etoposide (E), without (ADE) or with PSC-833 (ADEP). The incidence of complete remission was 75% with both regimens. Reversible grade 3 and 4 liver and mucosal toxicities were significantly more common with ADEP. Therapy-related mortality was 7% and did not differ by induction arm. Excess cardio-toxicity was not seen using high doses of D in ADE. The median DFS was 1.34 years in the ADE arm, and 1.09 years in the ADEP arm ($p = 0.74$, log rank test); the median OS was 1.86 years in the ADE arm and 1.69 years in the ADEP arm ($p = 0.82$). There is no evidence of a treatment difference within any identifiable patient subgroup. Inhibition of MDR1-Pgp-mediated drug efflux by PSC-833 do not improve clinical outcomes in younger patients with untreated AML. Potential explanations for the lack of benefit of MDR1-Pgp modulation by PSC-833 in AML include suboptimal modulation of efflux and increased treatment toxicity because of inhibition of clearance of anthracyclines via interference with MDR-Pgp-mediated hepatobiliary excretion or metabolism [71]. Pharmacokinetic interactions are generally unpredictable and some patients are probably under-dosed whereas others are over-dosed. Furthermore in contrast to CsA that inhibit ceramide glycosylation, PSC-833 has a different biological effect, raising ceramide level by stimulating *de novo* synthesis [60-72]. Third-generation inhibitors are designed specifically for high transporter affinity and low pharmacokinetic interaction. Inhibition of cytochrome P450

3A, which is responsible for many adverse pharmacokinetic effects of previous-generation inhibitors has generally avoided with the latest generation of inhibitors [30, 31]. The incorporation of zosuquidar, which is a potent and highly selective modulator of MDR1-Pgp in the randomized, placebo-controlled trial of the Eastern Cooperative Oncology Group 3999 failed to improve AML outcome in older patients. The lesson learned from this study is that the high selectivity for MDR1-Pgp chemical modulator may represent a functional restraint in clinical setting since other members of the ABC drug transporter family, such MRP1 or BCRP may act in concert to confer the multidrug resistance phenotype in AML cells overwhelming any potential benefit of zosuquidar as MDR reversing agent [73]. Biological and genetics studies show that clinical MDR is multi-factorial and an effective modulation may require targeting multiple drug transporter proteins which may be expressed in turn during chemotherapy treatment as an escape mechanism for AML cells to survive to the selective pressure condition [74].

Tackling the biological heterogeneity of the MDR phenotype which so far it is not fully deciphered in its genetic and biological complexity in tumors [75] by a safe and effective MDR reversing agent showing unrelated pharmacological effects, and no pharmacokinetic interactions with other drugs could represent an elusive perspective of cancer therapy. In contrast to the concept of high selective and MDR1-Pgp specific modulator, should be considered as medically relevant the MDR reversing agent with a certain grade of aspecificity such as quinine, quinidine and CsA. In principle, CsA should represent one of the best (and less expensive) modulator in tackling expression of MDR1-Pgp or unknown related drug resistance mechanisms pre-existing or emerging in tumor cells in response to chemotherapy. Clinical MDR is multi-factorial, and effective modulation may require targeting of multiple transport proteins of the ABC drug transporter family. Hence, the use of a single broad spectrum modulator it would be more efficacious in tackling the MDR phenotype of AML cells than MDR1-Pgp specific reversing agent. Furthermore, CsA in addition to its broad-spectrum MDR transporters reversing capability, shows other curative benefits. These include inhibition of cell growth [76] induction of apoptosis in at least some cell types [77] as well as anti-angiogenic effects [78]. In this context, a phase III trial conducted by the Southwest Oncology Group (SWOG) [54] and a multicenter randomized trial of the Leukemia Working Group of the Hellenic Society of Hematology [79] supported the combination of CsA with induction chemotherapy. This curative regimen improves outcomes in elderly people suffering from secondary AML without increasing drug toxicity and treatment-related mortality. In contrast, a phase III trial conducted by the United Kingdom Medical Research Council (MRC) did not support the use of CsA with induction chemotherapy [80]. The variations in CsA dosing along with the use of continuous infusion anthracycline in the SWOG study as opposed to the more standard bolus dosing used in the other two studies have made interpretation of the clinical utility of CsA difficult. Further studies are warranted to confirm the clinical benefits of CsA as a privileged MDR reversing agent by tailoring curative regimen in relapsed/refractory AML expressing MDR1-Pgp and related ABC transporter proteins as major mechanisms of drug resistance. Apart chemical modulators biological and genetics strategies have been experimented to render *de novo* susceptible MDR cells to cytotoxic drugs with tolerable safety profile. In this context we report and summarize in *Table 3* an array of biological agents which deserve more attention for their potential modulator capability of MDR1-Pgp in appropriated in *in vitro/in vivo* models.

PHARMACEUTICALS AS MODULATOR OF MDR1-Pgp

Collateral sensitivity reflects the paradoxical phenomenon of hypersensitivity observed in certain drugs where the MDR cells counterpart is found to be collaterally sensitive to membrane-active agents such as the calcium-channel blocker verapamil [81]. In recent years a number of already experimented drugs are proposed as a route to eradicate the MDR cell population in solid tumours and haematological malignancies. For example tiopronin (currently used for cystinuria) as chemical modulators may show a dual favorable properties: susceptibility of MDR cells to cytotoxic drugs and disabling MDR1-Pgp as multidrug transporter. The treatment of MDR cells with tiopronin led to instability of the MDR1 mRNA and consequently a reduction in MDR1-Pgp, despite functional assays demonstrating that tiopronin does not interact with MDR1-Pgp. Long-term exposure of MDR1-Pgp expressing cells to tiopronin sensitized them to doxorubicin and taxol, both MDR1-Pgp substrates. Treatment of MRP1-overexpressing cells with tiopronin led to a significant reduction in MRP1 protein. Synthesis and screening of analogs of tiopronin demonstrated that the thiol functional group was essential for collateral sensitivity, while substitution of the amino acid backbone altered but not destroyed specificity, pointing to future development of targeted analogs [82]. The calcium-channel blocker verapamil by interfering with MDR1 mRNA expression levels shows the ability of to kill MDR cells selectively over the parental cells from which they are derived [83]. Other compounds which include gemcitabine [84, 85] and rapamaycin [86] show the dual favorable properties of MDR reversing agents and preferential cytotoxicity towards MDR cell variants of tumor cells. These agents were experimented in clinical trials to improve anti AML activity of drugs included in standard and novel chemotherapy regimen. The results even controversial represent promising perspectives for AML therapy. Novel MDR reversing agents currently studied in older patients with refractory or relapsed AML include tipifarnib which is an oral, very potent, and highly selective farnesyl transferase inhibitors (FTIs) with a relatively low toxicity profile and

Table 3
Biological agents with MDR1- Pgp reversing ability

Biological agent	Mechanism for MDR reversing	Experimentations	References
Statins	MDR1-Pgp is predominately localized within cholesterol-rich plasma membrane microdomains, which are disrupted by cholesterol depletion. This suggests that statins and other inhibitors of the cholesterol biosynthetic pathway may be of value in modulating MDR1-mediated drug resistance in AML.	None	[60]
Transmembrane MDR1-Pgp peptides	Development of a panel of highly specific peptide inhibitors of MDR1-Pgp based on the structure of the transmembrane domains (TDM). These peptides are thought to exert their inhibitory action by disrupting the proper assembly of MDR1-Pgp	*In vitro*/animal model studies	[97]
Policlonal antibodies to rodent *mdr1* gene	Policlonal antibodies derived from immunization of mice with external sequences of the murine gene *mdr1* are capable of reverting the MDR phenotype *in vitro* and *in vivo*, without eliciting autoimmune response.	*In vitro*/animal model studies	[98]
Murine mAb MC57 and mAb MM12.10	CsA, its derivative SDZ PSC 833 and the semi-synthetic cyclopeptide SDZ 280-446 combined with mAb to MDR1-Pgp significantly affect the drug transport mechanism of human MDR cells	*In vitro*/animal model studies	[64]
Murine mAb UIC2	Inhibition of MDR1-Pgp function by blocking MDR1-Pgp in a transient conformation with no ATP bound	*In vitro* studies	[94]
Murine mAb HD37	Anti-CD19 murine mAb might chemo- sensitizes MDR1-Pgp cells by translocating of MDR1-Pgp into a compartment on the plasma membrane where it is no longer active.	*In vitro* studies	[95, 96]

possessing MDR1-Pgp inhibitory function in addition to its FTI activity [87]. As single agent tipifarnib exhibits modest activity in elderly adults with newly diagnosed AML. In contrast, tipifarnib administered in combination with etaposide synergizes the curative effect of concurrent administered anti cancer drug, very likely by MDR1-Pgp down-modulation. Based on preclinical synergy, a phase I trial of tipifarnib plus etoposide yielded 25% CR. However, Karp *et al.,* [88] matched the outcome of the curative regimen with the expression of RASGRP1 (which encodes the Ras-activating guanine nucleotide exchange factor) and *APTX* (which encodes the DNA excision repair protein aprataxin) genes. The results, show that AMLs with a RASGRP1/APTX ratio of more than 5.2 has a 78% CR rate. This study contains important indications in the search for the most effective way to use tipifarnib and addressing the utilization of innovative genetic strategies for personalized therapies in the treatment of elderly AML. Recently, Jawad *et al*., [89] using 34 primary AML samples, showed that the combination of GO and the FTI tipifarnib is successful at not only targeting the bulk cells but even more so the CD34+CD38 cell fraction under protective "niche like" conditions. This finding even tough emerging from an (accurate) analysis in *ex vivo* condition demonstrates that the stem cells progenitor which are quiescent and drug resistant may be susceptible to this novel therapeutic combination of a MDR reversing agent and GO. In order to identify the mechanisms underlying GO and calicheamicin-gamma1 resistance, flow cytometry-based single cell network profiling (SCNP) assays was utilized by Rosen *et al.,* [90] to study cellular responses of primary human AML cells to GO. In particular, these investigators found that (i) the extent of DNA damage is quantitatively impacted by CD33 expression and drug efflux activity (ii) DNA damage induced by calicheamicin-gamma1 is required for GO-induced cytotoxicity but not sufficient for effective cell kill and, (iii) a downstream anti-apoptotic pathways may function as relevant resistance mechanisms. Furtermore, Rosen *et al.,* [90] found that activated PI3K/AKT signaling is associated with GO resistance *in vitro* in primary AML cells and that investigational AKT inhibitor MK-2206 significantly sensitized various human AML cells to GO. Although future studies with larger numbers of patient specimens will be required to validate the biological function of MK-226 and quantify the contribution of AKT-mediated resistance to GO and calicheamicin-gamma1 in detail, the study of Rosen *et al.,* [90] highlight the potential of SCNP assays to differentiate AML samples based on underlying biology which might be relevant to therapeutic interventions.

BIOLOGICAL APPROACHES FOR MDR REVERSING PHENOTYPE

Recently new and sophisticated strategies which include genetics-biochemical intervention on MDR1-Pgp functional structure and linker-drug conjugate of ADC to inhibit drug efflux are proposed as a novel category of MDR reversing agents. To evade the MDR1-mediated resistance, Kotvun *et al*., [91] conjugated the highly cytotoxic maytansinoid DM1 via the maleimidyl-based hydrophilic linker (PEG4-Mal). Following uptake into target cells, conjugates made with the PEG4-Mal linker are processed to a cytotoxic metabolite which selectively kills MDR1-expressing cells in culture and xenograft human tumors. Other biological MDR reversing strategies may include mAbs disabling MDR1-Pgp by direct interaction with MDR1-Pgp extracellular domain [92-94] or interfering with MDR1-Pgp function by transferring this pathway via mAb CD19 binding into a compartment on the plasma membrane where MDR1-Pgp is no longer active [95, 96]. Furthermore, MDR1-Pgp-mediated drug resistance can be reversed by hydrophobic peptides that are high-affinity MDR1-Pgp substrates. Terasova *et al*., [97] developed a panel of highly specific peptide inhibitors of MDR1-Pgp based on the structure of the trans-membrane domains of the transporter. These peptides are thought to exert their inhibitory action by disrupting the proper assembly of MDR1-Pgp conferring MDR. The studies strongly suggest that potent and selective inhibitors of ABC transporters can now be developed solely on the basis of the primary structures of the target proteins. The newly synthesized MDR1-Pgp antagonists appear nontoxic drug resistant inhibitors that merit further development. Antibody -based approaches for the eradication of MDR cells is also proposed by Pawlak-Robin *et al*., [98] with the formulation of palmitoyl-peptides mimicking the external loops of the rodent *mdr1* gene product reconstituted in liposomes. The immunization of mice with this immunoconstruct elicited a strong immune-response in mice and sera from these mice were able to inhibit activity in L1210 MDR cells *in vitro*. Surprisingly, these palmitoyl -peptides, suspended either in PBS or in PBS alum or reconstituted in liposomes without alum, does not induce any auto-immune lesions in the kidney, liver, lung, adrenals and pancreas, up to 18 months after re-immunization. This finding address a novel method for MDR1-Pgp modulation that wait to be extended in in *vivo* model.

CONCLUSION AND PERSPECTIVES

By overviewing 12 years of literature concerning the pharmacology of GO, common issues emerge from clinical trials and *ex vivo* studies of AML treated with this first in class ADCs: (i) high level of expression of MDR1-Pgp in AML cells is associated with poor outcomes, (ii) attempts to improve the safety and efficacy of GO by inhibiting MDR1-Pgp function by various classes of MDR reversing agents are so far disappointing and none of such strategies has proved to be safe, effective and reproducible and, (iii) cytogenetics remains the most important prognostic feature of newly diagnosed AML. Three risk categories -*favorable, intermediate* and *unfavorable* risks are recognized based upon outcomes by chromosomal abnormalities in several large series of patients. In this context, Lin

and Levy [99] reported that the median survivals in each category are as follows: *favorable* risk, 7.6 years; *intermediate* risk, 1.3 years; and *unfavorable*, 0.5 years. Furthermore, 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 an *intermediate* prognosis, can now be further divided into a myriad of molecular subgroups, some of which are known to have significant prognostic implications [100]. Within the first year after approval, the FDA required a black box warning be added to GO packaging. The drug was noted to increase the risk of VOD in the absence of bone marrow transplantation. Later the onset of VOD was shown to occur at increased frequency in GO patients even following bone marrow transplantation. Common side effects of administration also included shivering, fever, nausea and vomiting. Serious side effects included severe myelosuppression (suppressed activity of bone marrow, which is involved in formation of various blood cells (found in 98% of patients), disorder of the respiratory system, tumor lysis syndrome, Type III hypersensitivity, venous occlusion, and death. A randomized phase III comparative controlled trial (SWOG S0106) was initiated in 2004 by Wyeth in accordance with the FDA accelerated-approval process. The study was stopped prior to completion due to worrisome outcomes. Among the patients evaluated, fatal toxicity rate was significantly higher in the GO combination therapy group *versus* the standard therapy group. Mortality was 5.7% with GO (6 mg/m2) and 1.4% without the agent [6]. However, an induction death rate of 5% to 7% is a feature of most induction therapies, and this was not observed in the large total experience of more than 2200 randomly assigned patients conducted by Burnett *et al*., [101] in MRC AML15 and MRC AML16 clinical trials. Because there is also emerging evidence for a daunorubicin dose effect in younger patients, the SWOG trial, which compared a daily dose of daunorubicin 60 mg/m2 with daunorubicin 45 mg/m2 plus GO in younger patients, could be interpreted as evidence for a benefit from GO, in that it compensated for the daunorubicin dose reduction. From the large randomized MRC AML15 and MRC AML16 experiences in more than 2200 randomly assigned patients across all age groups, it appear evident that GO at 3 mg/m2 administered simultaneously with daunorubicin (50 mg/m2) as part of induction therapy is safe, significantly reduces relapse risk, and improves OS [101]. To this latter regard 5/5 studies involving the use of low and/or and fractionated doses of GO (3 mg/m2) in day 1, 4 and 7 [16, 17, 45] in AML patients have found a benefit in newly diagnosed patients with *favorable* risk AMLs, and 4 of 5 have reported the same benefit in patients with *intermediate*-risk AML, as defined according to cytogenetic criteria. Since these patients represent the majority of AML patients, it would be important and justified to propose a re-approval of GO in AML patients with more favorable risk disease, in combination with cytabarine and anthracycline-based chemotherapy [102]. Whole genomic sequences performed in order to determine the mutational spectrum associate with primary tumor and relapse genomes from 8 AML patients, allowed Ding *et al*., [103] to discover novel, recurrently mutated genes (*e.g*. *WAC*, *SMC3*, *DIS3*, *DDX41*, and *DAXX*) in AML. They also found two major clonal evolution patterns during AML relapse: 1) the founding clone in the primary tumor gained mutations and evolved into the relapse clone, or 2) a subclone of the founding clone survived initial therapy, gained additional mutations, and expanded at relapse. In all cases, chemotherapy failed to eradicate the founding clone. The comparison of relapse-specific *versus* primary tumor mutations in all 8 cases revealed an increase in transversions, probably due to DNA damage caused by cytotoxic chemotherapy. These data demonstrate that AML relapse is associated with the addition of new mutations and clonal evolution, which is shaped in part by the chemotherapy that the patients receive to establish and maintain remissions.

A number of reports have addressed the role of specific genetic impact in AML pharmacotherapy. These include ABCB1 and ABCB2 drug transporter polymoprphisms which might be involved on clinical outcomes of AML via a number of mechanisms. Shaffer *et al*., [31] hypothesize that efficiency of transporter function could be increased via decreased binding to inhibitors or, on the other hand, dysfunctional transporters could lead to increased toxicity due to decreased export of chemotherapy drugs from normal tissues, particularly bone marrow cells.

Furhermore, clinical implications of CD33 single-nucleotide polymorphisms (SNP) might impact in pediatric patients with AML treated with GO-based therapy. To this regard, Mortland *et al*., [104] suggest that genetic variations at SNPs level in CD33 could impact clinical outcome of GO-based therapy in pediatric AMLs. Although the toxic effects of immunoconjugates containing calicheamicin-gamma1 are in many cases antigen-specific, it is reported that GO elicits potent antitumor activity in antigen-independent manner [105]. Furthermore, free calicheamicin-gamma1 not sparing normal tissues may induce severe toxicity [8]. Therefore, it is possible that non-specific drug release through hydrazone linker instability contributes to low safety profile of GO [106]. Nonetheless, cancer therapy is moving rapidly and irreversibly in the direction of personalized therapy with better characterization of disease subsets and development of selective, target-specific drugs that are either highly effective alone or complement current therapeutic strategies. To this regard several new therapeutic modalities can be explored using existing inhibitors. To this regard, pharmacogenomics analysis has identified the subset of patients with a defined RASGRP1/APTX ratio showing high efficacy of FTI in

reversing the MDR phenotype of AML cells and improving the outcomes of patients treated with etoposide-containing curative regimen [88]. Furthermore, most of the clinical trials are carried out in patients with prior therapies, in whom acquired resistance is likely to have developed through multiple mechanisms suggesting the hypothesis of preventing, rather than fighting, MDR cancer [107]. Hence an appropriated curative regimen requires an *ex vivo* examination of AML blasts for expression and function of MDR mechanism in its dynamic presentation during immunochemotherapy treatment to identify the best and safety modality to accumulate into target cells the conveyed payload and concurrent drugs. The lesson learned for this first-in-class antibody ADCs pave the way for next generations of immunoconjugates [108]. A consistent asset of promising new ADCs are currently investigated in clinical trials and two of them, Brentuximab vedotin [10] and T-DM1 [12], have been recently approved for commercial distribution in US by FDA. This second generation ADCs employ linkers of protease-cleavable peptides. The drugs are appended *via* a valine-citrulline (*vc*) dipeptide linkage designed for high stability in serum and conditional cleavage and putative release of fully active drugs by lysosomal cathepsins. This drug linker system shows high stability *in vitro* and *in vivo* and when applied to multiple mAbs, the resulting ADCs are selectively potent and effective against cognate antigen-positive tumor cells and tumor xenografts. This linker system was also efficiently applied to anti CD30 antibodies in single chain fragment variable (scFv) format (scFv diabodies were conjugated with 4 equivalents MMAE or MMAF, *via* a protease-cleavable dipeptide linker, to create the conjugates diabody-vcE4 and diabody-vcF4, respectively) [109]. This third generation of ADCs may offer a potential way to circumvent some of pharmacokinetics limitations of GO due to non-specific drug release through linker instability that may contributes to the severe adverse effects observed during immunochemotherapy. This novel format of antibody drug conjugate may be designed for selective delivering curative payload to AML thus sparing normal tissues. For instance scFv anti CD33 antibody formulated by genetic engineering manipulation in diabody [110] or small immune proteins (SIP) [111] format could be conjugated with calicheamicin-gamma1 or similarly to Brentuximab vetotin with 4 equivalents MMAE or MMAF, *via* a protease-cleavable dipeptide linker [10, 109]. This novel designed ADC which can be categorized as biosimilar product may be of great help in therapeutic treatment of AML as complementary version of GO [112].

Conflicts 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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