Cancer Letters format (between 3000 – 3500 words)

Title (not exceeding 185 characters including spaces)

High affinity and covalent-binding microtubule stabilizing agents show activity in chemotherapy-resistant acute myeloid leukemia cells.

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Abstract (not exceeding 185 words excluding spaces)

Treatment failure in acute myeloid leukemia (AML) is frequently due to the persistence of a cell population resistant to chemotherapy through different mechanisms, in which a drug efflux via ATP-binding cassette (ABC) proteins, specifically P-glycoprotein, is one of the best recognized. However, the large number of failed clinical trials employing inhibitors for these transporters has demonstrated the necessity to adopt different strategies. We hypothesized that microtubule targeting compounds presenting high affinity or covalent binding could overcome the effect of the ABC-transporters. We therefore evaluated the activity of the high-affinity paclitaxel analogue chitax-40 (CTX-40) as well as the covalent binder zampanolide (ZMP) and novel ZMP-derivatives in AML cells. All molecules were active in chemosensitive and chemoresistant cell lines overexpressing P-glycoprotein, being ZMP and CTX-40 the most effective. Moreover, ZMP or CTX-40 in combination with daunorubicin showed synergistic killing without increased *in vitro* hematopoietic toxicity. In addition, we demonstrated that ZMP and CTX-40 are active in the progenitor and differentiated AML populations from a primary sample. In sum, our data indicates that high

affinity and covalent-binding anti-microtubule agents are active in otherwise chemotherapy resistant AML cells.

Keywords (maximum of 6)
microtubules
leukemia
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1. Introduction

Acute myeloid leukemia (AML) is a clonal disorder characterized by the inhibition of differentiation with the resulting accumulation of immature cells in the bone marrow and/or peripheral blood (Ferrara and Schiffer 2013). The current treatment of most common types of AML has hardly changed over the past three decades, and is composed of induction chemotherapy (usually a combination of cytarabine and an anthracycline), followed by either consolidation chemotherapy or allogeneic stem cell transplantation (Roboz 2012). Although this treatment leads to a complete remission in the majority of patients, only 40 % of younger than 60 years and 10 to 20 % of older patients remain in remission (Peloquin, Chen et al. 2013).

Treatment failure it has been frequently associated to the persistence of cell population that is inherently resistant to chemotherapeutic agents (Tallman, Gilliland et al. 2005, Ishikawa, Yoshida et al. 2007). One of these resistance mechanisms is due to an increased cellular efflux of drugs via transmembrane proteins of the ATP-binding cassette (ABC) family, including P-glycoprotein (P-gp), multidrug resistance-associated protein 1, and breast cancer resistance protein (Wulf, Wang et al. 2001, van den Heuvel-Eibrink, van der Holt et al. 2007). The main approach to overcome the efflux of chemotherapy agents in AML has involved the co-administration of competitive inhibitors of these pumps. However, the large number of failed clinical trials involving ABC family inhibitors has demonstrated the necessity to adopt different strategies (Libby and Hromas 2010).

Development of tubulin-binding agents with high binding affinity has been proposed as an alternative strategy to overcome the transport efflux (Buey, Calvo et al. 2007, Yang, Barasoain et al. 2007, Matesanz, Barasoain et al. 2008). In a proof-of-principle experiment, we previously reported that a taxane-derivative with 500-fold higher affinity than paclitaxel, CTX-40, can effective overcome efflux pumps, including P-gp (Matesanz, Barasoain et al. 2008, Cai, Lu et al. 2013). Likewise, the tubulin covalent-binding drug zampanolide (ZMP) showed activity in one breast cancer cell line overexpressing drug pumps (Field, Pera et al. 2012), suggesting that this could also be a valid strategy for killing chemoresistant cells.

Herein, we determined the anti-leukemic effects of CTX-40, ZMP and a series of novel synthetic structurally simplified ZMP-derivatives (Figure 1) in chemotherapy-resistant AML cell lines and in an AML primary sample. We also characterized the effect of their combination with the anthaycline daunorubicin, as well as their toxicity to human hematopoietic progenitors and stem cells (HPSCs).

2. Materials and methods

2.1. Reagents

Full details of the synthesis and characterization of CTX-40, ZMP, MJ-75, *epi*-MJ-75, MJ-SP-15, MJ-88, MJ-90, MJ-91, MJ-97, MJ-98, MJ-103 and MJ-105 can be found in the Supplementary Data. Paclitaxel and vinblastine were obtained from Sigma, and cytarabine and daunorubicin were obtained from the Memorial Sloan-Kettering Cancer Center pharmacy. All compounds were dissolved in dymethil sulfoxide (DMSO) (Sigma) at 20 mM as a stock solution.

2.2. Cell lines and primary specimens

Human umbilical cord blood (CB) from healthy full-term pregnancies was provided by the New York Blood Center. Human $CD34^+$ cells were isolated from Ficoll-separated mononuclear CB cells using the MiniMACS CD34 isolation kit (Milteny Biotech) as previously described (Chung, Morrone et al. 2005).

AML patient sample was collected under a Memorial Sloan-Kettering Cancer Center Institutional Review Board and ethics committee-approved clinical protocol with informed consent. The examined mutations and cytogenetic abnormalities were determined via fluorescence *in situ* hybridization (FISH), karyotyping and DNA sequencing (Flt3, NPM1, CEPBα, KIT). Samples were centrifuged over Ficoll-Paque PLUS (GE Healthcare) step gradients (2,000 *g* for 30 min), yielding mononuclear cells. Then, $CD34^+$ cells were isolated using MiniMACS CD34 isolation kit.

The murine MS-5 bone marrow-derived stromal cell line was grown in α-modified essential medium (α-MEM) containing 12.5 % FCS (Hyclone) and 12.5 % horse serum (Hyclone), 1 % penicillin and streptomycin, 200 mM glutamine, 1mM monothioglycerol (Sigma Cell Culture) and 1 μM hydrocortisone (Sigma).

The human AML cell lines MV4-11, HL-60 and KG-1a, and the acute lymphoblastic leukemia (ALL) cell line Reh were purchased from American Type Culture Collection (ATCC, Manassas, VA). MV4-11 and HL-60 were cultured in Iscove's modified Eagle medium (MSKCC Media Facility), containing 10 % FCS, 200 mM glutamine and 1 % penicillin and streptomycin (IMDM complet). KG-1a were cultured in IMDM complete medium with 20 % FCS. The ALL cell line CCRF-CEM and their resistant clone CCRF-CEM/VBL were cultured in RPMI-1640 medium (MSKCC Media Facility) containing 10 % FCS, 200 mM glutamine and 1 % penicillin and streptomycin. The CCRF-CEM/VBL cell

line was cultured in presence of 0.5 µM vinblastine until 7 days before the experiments. All cell lines were incubated at 37 ºC / 5 % CO_2.

2.3. In vitro *toxicity studies*

Growth inhibition 50 (GI_{50}) values for the tested molecules were determined by a fluorescence assay using 7-Hydroxy-3H-phenoxazin-3-one 10-oxide (alamar blue, Invitrogen) according to the manufacturer's protocol after 72 h of drug incubation period.

2.4. Cell cycle assays

Cell cycle fractions were determined by propidium iodide nuclear staining. Briefly, cells were harvested, washed in PBS, fixed with 70 % ethanol, and incubated with propidium iodide/RNase buffer (BD Bioscience) for 24 h at 4 ºC. Data were collected on a BD LSR Fortessa fluorescence-activated cell analyzer using BD FACS Diva software and analyzed using FlowJo version 10.0.6 (Tree Star Inc.).

2.5. Immunoblot analyses

Protein concentrations were determined using the BCA kit (Pierce Biotechnology) according to the manufacturer's instructions. Protein lysates (15 - 30 µg) were electrophoretically resolved by SDS/PAGE, transferred to nitrocellulose membrane, and probed with the indicated primary antibodies: Anti-Mdr-1 (1:500, sc-55510; Santa Cruz). Equal loading of the protein samples was confirmed by parallel western blots for β-actin (1:50,000, ab822750; Abcam). We used ECL-Enhanced Chemiluminescence Detection System (GE Healthcare Biosciences, Pittsburgh, PA) according to the manufacturer's instructions and the blots were visualized by autoradiography. Quantitative densitometry analysis of western blot bands was performed employing Image J version 10.2 software (NIH). The normalized relative densities were calculated relative to the expression of β-actin.

2.6. *Real-time qPCR*

Total RNA was extracted from 5 x 10^6 cells with the use of the RNeasy Mini Plus kit (Qiagen) and eluted in RNAse-free water. cDNA was synthesized using high capacity RNA-to-cDNA kit (Applied Biosystems). Primer sequence for MDR-1 was published in (Lobert, Hiser et al. 2010). SYBR Green FastMix was from Quanta BioSciences. The thermal cycling parameters are as follows: (…).

2.7. Caspase assays

Caspase-3 and -7 activity was determined employing the Apo-ONE caspase 3/7 assay (Promega) following the manufacturer's instructions and measuring the fluoresence emission in a Synergy4 microplate reader (BioTek). Caspase activity was normalized by the cell number determined by alamar blue. Caspase-9 inhibitor I was from Calbiochem and caspase-8 inhibitor was from G-Biosciences.

2.8. Colony-forming unit (CAFC) and cobblestone area-forming cell (CAFC) assays for hematopoietic stem and progenitor (HSPC) cells

For the CAFC assays, 8,000 cord blood $CD34^+$ (CB-$CD34^+$) cells were incubated with compound for 72 h at 37 ºC / 5 % CO_2 in QBSF-60 (MSKCC Media Facility), 1 mM monothioglycerol, 2 mM glutamine, 20 ng/mL c-kit ligand (KL), thrombopoietin (TPO) and Flt3 ligand (FL). KL was obtained from Peprotech, TPO (…) and FL (…). After the incubation period, the compounds were washed out and the colony-forming assays were performed in triplicate in a 35 mm plate (1,000 cells per well) using 1.2 % methylcellulose (Dow Chemical), 30 % FCS, 1 mM monothioglycerol (Sigma), 2 mM glutamine, 0.5 mM hemin (Sigma), 20 ng/mL interleukin 3 (IL-3), granulocyte colony-stimulating factor (G-CSF), and KL and 6 U/mL erythropoietin (Epo). IL-3 was from Peprotech, G-CSF was obtained from Amgen and Epo from Ortho Biotech. Samples were incubated at 37 ºC / 5 % CO_2. Colonies were scored 14 days after plating.

CAFC assays were performed by plating 2,000 CB-$CD34^+$ 72 h preincubated cells on to MS-5 monolayers in T12.5 tissue-culture flasks (Becton Dickinson) in duplicate. Weekly half of the medium and cells were removed and replaced with fresh medium (demidepopulation). A cobblestone was defined as an instance of at least 10 six tightly packed cells beneath the MS-5 stromal monolayer (Breems, Blokland et al. 1994).

2.9. CAFCs for leukemic stem cells

MS-5 mouse bone marrow-derived stromal cells were plated in 96-well format (20,000 cells per well in α-MEM) and kept at 37 ºC / 5 % CO_2. One day later, $CD34^+$ preincubated primary-leukemic cells were added in 100 µL of fresh co-culturing medium (α-Eagle's minimum essential medium, 12.5 % horse serum, 12.5 % FBS, 200 mM glutamine, 1 % penicillin and streptomycin, 1 mM monothioglycerol and 1 µM hydrocortisone) at a density determined in order to generate 10 cobblestones per well after 2 weeks (Moore, Dorn et al.

2007) in neutral control wells. The co-cultures were then maintained and assessed for cobblestone at week 2.

2.10. Drug combination analysis

Drug interaction evaluation was assessed employing the combination index (CI) equation of Chou and Talalay (Chou and Talalay 1984) and Berenbaum (Berenbaum 1985): $CI = (D_1/Dx_1) + (D_2/Dx_2)$. A CI value equal to one indicates additivity, values less than one indicate synergy, and values greater than one indicate antaganosim. Doses D_1 and D_2 correspond to those used in combination, and the doses Dx_1 and Dx_2 correspond to the amounts of each drug given alone that would produce the same response as obtained with the combination. In order to calculate the concentration of drug needed for a given response on its own, the following equation was used: $\text{Dose } (x) = GI_{50} \times (\text{max} - \text{response} / \text{response} - \text{min})^{1/\text{hillslope}}$. GI_{50} and values for minimum (min), maximum (max) and hillslope were obtained using GraphPad Prism version 6.0b software.

3. Results

3.1. High affinity and covalent tubulin-binding agents inhibited proliferation of drug-resistant leukemic cells

We determined the activity of high affinity and covalent binding microtubule stabilizing agents in in a panel of six human leukemia cell lines that included the P-gp overexpressing cell lines KG-1a (acute myeloid leukemia, AML) (Koeffler, Billing et al. 1980, Wang, Wang et al. 2014) and CCRF-CEM/VBL (acute lymphoid leukemia, ALL) (Bacherikov, Chou et al. 2004) (Supplementary Figure 1). We compared their effect against clinically used chemotherapy drugs for leukemia treatment, daunorubicin and cytarabine. We used paclitaxel as control for CTX-40 since this compound is a paclitaxel derivative with 500 fold higher binding affinity for tubulin. We exposed cells to these compounds for 72h and measured viability by a metabolic dye reduction assay (alamar blue). We found that all the compounds reduced leukemia cell proliferation (Table 1 and 2). Daunorubicin and paclitaxel increased their GI_{50} in P-gp overexpressing cells by 10 and 60 fold, respectively ($p < 0.05$, Table 1), while cytarabine, maintained its effect (Table 1). On the contrary, ZMP showed sub-nanomolar GI_{50} values in all the P-gp-expressing cell lines, and CTX-40 increased its GI_{50} value, although within the nanomolar range, only in CCRF-CEM/VBL cells (Table 1). All ten ZMP-derivatives reduced cell proliferation at the micromolar range and retained activity in PgP-expressing resistant cell lines (Table 2). Taken together, these results demonstrate that compounds with high affinity or covalent binding are able to circumvent the effect of pump efflux.

3.2. ZMP and CTX-40 induced cell cycle arrest and apoptosis in AML cells

To further characterize the anti-leukemic effect of the compounds ZMP and CTX-40, we exposed the sensitive cell line MV4-11 and the resistant cell line KG-1a to three concentrations of drugs for 12 and 24h, and measured cell cycling by DNA deconvolution. We found that ZMP and CTX-40 induced cell cycle arrest characterized by an increased of the fraction of cells in G_2/M phase and a decreased of the cell fraction at G_0/G_1 (Figure 2). The cycle arrest in MV4-11 occurred at 12 hours (Figure 2a), while in the P-gp overexpressing cell line, KG-1, the effect was more evident at 24 hours (Figure 2b). To determine whether G_2/M arrest was followed by cell death, we analyze the induction of apoptosis by determining the activation of caspase-3 and -7. We found that both compounds induced activation of caspase-3/-7 (Figure 3). Moreover, in presence of the caspase-8 or -9 inhibitors this activity was notably reduced (Figure 3a and b), suggesting

that ZMP and CTX-40 induce apoptosis in MV4-11 and KG-1a cells through engaging intrinsic and extrinsic apoptotic pathways.

3.3. ZMP and CTX-40 synergized with daunorubicin in MV4-11 and KG-1a cells

To determine whether ZMP and CTX-40 synergizes with daunorubicine, we exposed chemoresistant (KG-1a) and chemosensitive (MV4-11) AML cells to the concurrent and sequential combination of these drugs and measure their anti-proliferative effect. The combinatorial effect was determined by the combination index (CI) (Chou and Talalay 1984, Berenbaum 1985), where CI values less than 1.0 indicates a synergistic effect between two drugs. We found that the combinations of daunorubicine with ZMP and CTX-40 in KG-1a and MV4-11 cell lines were synergistic (Table 3). Remarkably, these results indicated a statistically significant effect for the combination even when ZMP and CTX-40 were administered at concentrations four-time lower than their GI_{50} (Table 3). There were not differences on the synergistic effect when drugs were administered sequentially (i.e. daunorubicin for 24 h followed by ZMP or CTX-40) (Supplementary Table 1). Notably, the combination of ZMP and CTX-40 with daunorubicin was synergistic even in the chemoresistant AML cell line KG1-a, suggesting a potential way to circumvent anthracycline resistance.

3.4. In vitro *toxicity of ZMP and CTX-40 toward $CD34^+$ normal hematopoietic cells resulted equivalent to cytarabine*

To determine the effect of ZMP and CTX-40 on human normal hematopoietic stem cells and human normal hematopoietic progenitor cells, we performed colony-forming unit (CFU) and cobblestone area-forming cell (CAFC) assays, respectively. In CFU assays cord blood (CB)-$CD34^+$ cells are cytokine-stimulated to differentiate into erythroid cells, granulocytes, macrophages and megakaryocytes. In CAFC assays, hematopoietic stem cells (HSC) are recognized *ex vivo* via the formation of the so called “cobblestone areas” (the burrowing of HSC beneath a monolayer of bone marrow fibroblasts, that results in the formation of phase-contrast dark areas cells tightly associated) (Eldjerou, Chaudhury et al. 2010). In the CFU assays CB-$CD34^+$ cells were preincubated 72 h with ZMP, CTX-40 and cytarabine, cells were later washed and cultured for 2 weeks. Pretreatment with ZMP and CTX-40 resulted in the inhibition of colony formation in a concentration dependent manner as it was observed with the cytarabine pretreatment (Figure 4a left). All 3 compounds induced a higher acute inhibition of the erythroid (Ery) linage compared to the

granulocyte/macrophage (GM) linage. Lower doses (0.15 and 0.3 nM) of CTX-40 induced a slightly highest inhibitory effect than ZMP, however both compounds exhibited similar activities at higher concentrations (0.6 and 1.2 nM). It was also observed an increase in the frequency of the primitive erythroid progenitor cells BFU-e (burst-forming unit – erythroid) over the later-stage erythroid progenitor cells CFU-e (colony-forming unit – erythroid) (Figure 4a right) indicative of a higher sensitivity of the more differentiated erythroid progenitor for the tested compounds. Nevertheless, the effects of both CTX-40 and ZMP on human hematopoietic progenitors cells were found similar to those of the clinically approved drug cytarabine.

In CAFC assays, in which the CB-$CD34^+$ cells were pulse treated for 72 h prior to seeding, ZMP and CTX-40 showed similar inhibitory effect as cytarabine in all tested concentrations (Figure 4b left). The cobblestones observed in the pretreated samples with cytarabine, ZMP or CTX-40, presented equivalent morphologies than those observed in the control group (Fig. 4b right).

3.5. ZMP and CTX-40 showed activity against AML-patient derived $CD34^+$ leukemic cells.

To determine how ZMP and CTX-40 would affect stem cell-like and differentiated leukemic cell populations, we evaluated the effect of ZMP and CTX-40 in $CD34^+$ cells from an AML patient with Flt3-ITD mutation.. AML patient-derived $CD34^+$ cells were treated fro 72 h with ZMP or CTX-40 (vs. vehicle) and, after drug washout, seeded in a MS-5 stroma cell layer. After two weeks of co-culture, cobblestones and suspension cells were scored. ZMP and CTX-40 proved to be equally potent toward the AML-tumor bulk cycling suspension cells compared to the sensitive or resistant leukemic cell lines employed in the study, showing GI_{50} of 0.3 and 0.8 nM for ZMP and CTX-40, respectively. ZMP resulted to be slightly more effective than CTX-40 against the leukemic stem cell fraction (Figure 5a and 5b).

4. Discussion

In the present study, we have demonstrated that microtubule-targeting agents (MTAs) with high binding affinity or covalent binding could effectively avoid pump-efflux from AML cells, a major cause of treatment failure in AML patients. Our results demonstrate that the marine MTA with covalent binding ability ZMP, and the synthetic paclitaxel derivative with high binding affinity CTX-40, have a subnanomolar killing activity on AML cell lines as well as on patient-derived bulk and leukemic stem cells. Both compounds preserved their activities in cell lines overexpressing P-gp and synergized with the antileukemic drug daunorubicin.

ZMP and CTX-40 were the most active molecules in inhibiting cell proliferation with GI_{50} values in the subnanomolar range, thus around 100 folds more potent than cytarabine. We obtained a resistance ratio (R/S), i.e. GI_{50} (resistant)/GI_{50} (sensitive), in AML cell lines of around 650 for paclitaxel and 13 for CTX-40, making CTX-40 50-times more active than paclitaxel. Collectively, our results indicated that CTX-40 is less affected by P-gp-mediated resistance at cellular level than its parental compound paclitaxel, and that the effect of ZMP and its novel derivatives are not decreased due the efflux transport.

All the novel ZMP derivatives tested showed antileukemia activity with GI50s from high nanomolar to low micromolar range. This variation in their potency is in agreement with the work published by Prota *et al.* (Prota, Bargsten et al. 2013) that demonstrated the requirement of the N-acyl-hemiaminal of ZMP to interact with the M-loop of β-tubulin to stabilize the microtubules. In our study, the most active ZMP derivatives were MJ-75 and *epi*-MJ-75 that preserved both the hydroxyl group in C20 responsible of the formation of a hydrogen bond with the M-loop.

Although ZMP and CTX-40 decreased the number of normal hemopoietic colonies, this effect was comparable to that induced by cytarabine. More remarkable was the effect of these drugs in killing leukemic cells from an AML patient. This patient presented an activating mutation of the FML-like tyrosine kinase 3 (Flt3), that are found in 30% of all AML cases (Kiyoi, Towatari et al. 1998) and are associated with an aggressive disease phenotype and poor outcomes (Patel, Gonen et al. 2012). We showed here that ZMP and CTX-40 were able to kill the tumor bulk as well as the quiescent leukemic stem cell population.

In sum, we showed here that is feasible to overcome the effect of efflux pumps affecting chemotherapy drugs, by employing compounds with higher affinity or covalent binding to

tubulin. Our study presents ZMP and CTX-40 as promising candidates for *in vivo* evaluation to validate their possible therapeutic use in AML.

Conflict of interest

We declare that we have no conflict of interest.

Acknowledgments

Supplementary material

- Synthesis of the ZMP derivatives
- Supplementary Figure 1
- Supplementary Table 1

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Figure Legends

Figure 1

Chemical structures of the compounds employed in the study. ZMP, zampanolide.

Figure 2

Cell cycle analysis of MV4-11 (A) and KG-1a (B) cells treated with vehicle, zampanolide (ZMP) or CTX-40 for 12 or 24 hours. The abundance of cells in each cell cycle phase is represented as a percentage of the total.

Figure 3

Caspas-3 and -7 activity (RLU) determined in MV4-11 (A) and KG-1a (B) exposed to vehicle, zampanolide (ZMP) or CTX-40 for 24 hours, in presence or absence of caspase-9 or caspase-8 inhibitor.

Figure 4

In vitro toxicity of zampanolide (ZMP) and CTX-40 on hematopoietic progenitor and stem cells. (A) Colony formation of 1,000 pulse-treated (72 h) CB-$CD34^+$ stem/progenitor cells with cytarabine (CYT), ZMP or CTX-40. On right, images of the observed colonies at week 2. (B) The effects of CYT, ZMP or CTX-40 on cobblestone formation of CB-$CD34^+$ cells. On right, representative images of the observed cobblestones at week 5. Scale bars, x μM.

Figure 5

In vitro toxicity of zampanolide (ZMP) and CTX-40 on a patient-derived $CD34^+$ leukemic cells. (A) The images depict representative week 2 cobblestone formation of primary AML patient $CD34^+$ cells in MS-5 cocultures. The white arrows indicate primary-$CD34^+$ tumor-bulk cells, and the black arrows indicate leukemic stem cell-cobblestones. (B) Effect of ZMP and CTX-40 on inhibiting proliferation of primary-$CD34^+$ tumor-bulk cells (curve) and leukemic stem cell-cobblestones (bars). Scale bars, x μM.

Supplementary Figure 1

Transcript abundance (fold change relative to MV4-11) of MDR-1 in the leukemia cell lines employed in the study, determined by RT-qPCR (A). PCR products were analyzed using 1 % agarose gel electrophoresis.