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Original Articles

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

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ABSTRACT

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 drug efflux via ATP-binding cassette (ABC) proteins, specifically P-glycoprotein, is one of the most recognized. However, disappointing results from clinical trials employing inhibitors for these transporters have demonstrated the need to adopt different strategies. We hypothesized that microtubule targeting compounds presenting high affinity or covalent binding could overcome the effect of ABC transporters. We therefore evaluated the activity of the high-affinity paclitaxel analog CTX-40 as well as the covalent binder zampanolide (ZMP) in AML cells. Both molecules were active in chemosensitive as well as in chemoresistant cell lines overexpressing P-glycoprotein. Moreover, ZMP or CTX-40 in combination with daunorubicin showed synergistic killing without increased *in vitro* hematopoietic toxicity. In a primary AML sample, we further demonstrated that ZMP and CTX-40 are active in progenitor and differentiated leukemia cell populations. In sum, our data indicate that high affinity and covalent-binding anti-microtubule agents are active in AML cells otherwise chemotherapy resistant.



## 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 [1]. 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 [2]. Although this treatment leads to a complete remission in the majority of patients, only 40% of patients younger than 60 years and 10–20% of older patients remain in remission [3].

Treatment failure has been frequently associated with the persistence of a cell population that is inherently resistant to chemotherapeutic agents [4,5]. One of these resistance mechanisms is 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 [6,7]. The main approach to overcoming 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 [8].

Development of microtubule stabilizing agents with high binding affinity has been proposed as an alternative strategy to overcome the transport efflux [9–11]. In a proof-of-principle experiment we previously reported that a taxane-derivative with 500-fold higher affinity than paclitaxel, CTX-40, can effectively overcome efflux pumps including P-gp [11,12]. Likewise the tubulin covalent-binding drug zampanolide (ZMP) showed activity in one breast cancer cell line that overexpressed efflux pumps [13],

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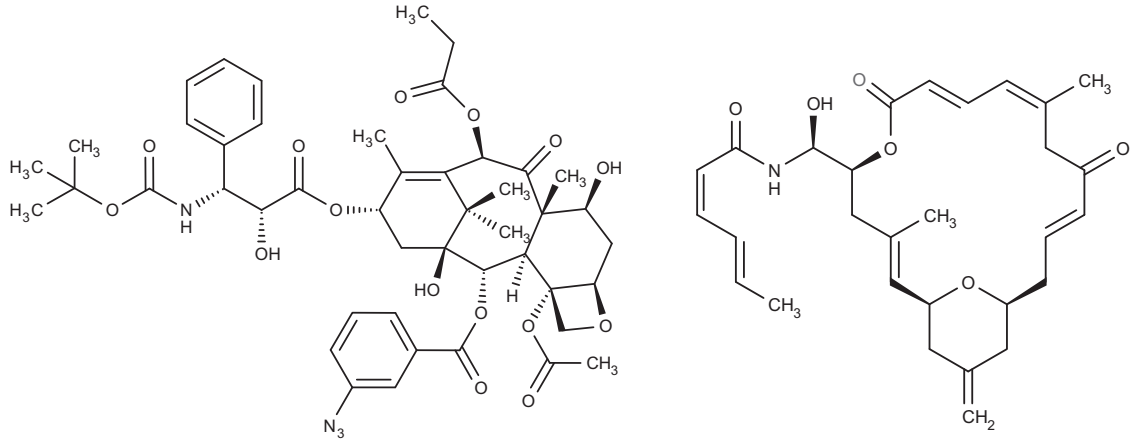


**Fig. 1.** Chemical structures of the compounds employed in the study. ZMP: zampanolide.

suggesting that this could also be a valid strategy for overcoming chemoresistance.

Here, we determined the anti-leukemic effects of CTX-40 and ZMP (Fig. 1) in chemotherapy-resistant AML cell lines and in an AML primary sample. We also characterized the effect of their combination with the anthracycline daunorubicin, as well as their toxicity to human hematopoietic progenitors and stem cells (HPSCs).

## Materials and methods

### *Reagents*

CTX-40 was synthetized as described in Cai et al. [12]. Zampanolide was synthetized following the procedure described by Zurwerra et al. [14]. 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 dimethyl sulfoxide (DMSO) (Sigma) at 20 mM as a stock solution.

### *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 (Miltenyi Biotec) as previously described [15].

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 (2000 *g* for 30 min), yielding mononuclear cells, and $CD34^+$ cells were isolated using MiniMACS CD34 isolation kits.

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, 1 mM 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 (Manassas, VA). MV4-11 and HL-60 were cultured in IMDM (MSKCC Media Facility), containing 10% FCS, 200 mM glutamine and 1% penicillin and streptomycin. KG-1a was cultured in IMDM medium with 20% FCS. The ALL cell line CCRF-CEM and its vinblastine-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 the presence of 0.5 μM vinblastine until 7 days before the experiments. All cell lines were incubated at 37 °C/5% $CO_2$.

### 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.

### *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 °C. Data were collected on a MACSquant fluorescence-activated cell analyzer and analyzed using FlowJo version 10.0.6 (Tree Star Inc.).

### *Real-time qPCR*

Total RNA was extracted from $5 \times 10^6$ 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). The primer sequence for MDR-1 was published in [16]. SYBR Green FastMix was from Quanta BioSciences.

### *Caspase assays*

Caspase-3 and -7 activity was determined employing the Apo-ONE caspase 3/7 assay (Promega) following the manufacturer's instructions with measurement of fluorescence 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.

### *Colony-forming unit (CFU) and cobblestone area-forming cell (CAFC) assays for hematopoietic stem and progenitor (HSPC) cells*

For the CFU assays, 8000 cord blood $CD34^+$ ($CB\text{-}CD34^+$) cells were incubated with compound for 72 h at 37 °C/5% $CO_2$ in QBSF-60 (MSKCC Media Facility), 1 mM monothioglycerol, 2 mM glutamine, 20 ng/mL c-kit ligand, thrombopoietin and Flt3 ligand. After the incubation period, the compounds were washed out and the colony-forming assays were performed in triplicate in a 35 mm plate (1000 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 (Peprotech), granulocyte colony-stimulating factor (Amgen), c-kit ligand and 6 U/mL erythropoietin (Ortho Biotech). Samples were incubated at 37 °C/5% $CO_2$. Colonies were scored 14 days after plating.

CAFC assays were performed by plating 2000 $CB\text{-}CD34^+$ 72 h preincubated cells onto 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. A cobblestone was defined as an instance of at least eight tightly packed phase-dark cells beneath the MS-5 stromal monolayer [17].

### *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 maintained at 37 °C/5% $CO_2$ for 24 h, after which $CD34^+$ 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 to generate 10 cobblestone areas per well after 2 weeks [18] in neutral control wells. The co-cultures were then maintained and assessed for cobblestone area formation at week 2.