Published in final edited form as:

Cancer Lett. 2015 November 1; 368(1): 97–104. doi:10.1016/j.canlet.2015.07.038.

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.

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Conflict of interest

We declare that we have no conflict of interest.

Keywords

Microtubules; Chemotherapy; Resistance; Acute myeloid leukemia (AML); P-glycoprotein

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

Here, we determined the anti-leukemic effects of CTX-40 (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 synthesized as is described in Cai et al. [12]. Zampanolide was synthesized 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 (Milteny Biotech) 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, CEPB α , 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 μ M 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 Iscove's modified Eagle medium (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 μ M vinblastine until 7 days before the experiments. All cell lines were incubated at 37 °C/5% CO₂.

In vitro toxicity studies

Growth inhibition 50 (GI₅₀) 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 BD LSR Fortessa fluorescence-activated cell analyzer using BD FACS Diva software and analyzed using FlowJo version 10.0.6 (Tree Star Inc.).

Real-time qPCR

Total RNA was extracted from 5×10^6 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-CD34⁺) cells were incubated with compound for 72 h at 37 °C/5% CO₂ 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% CO₂. Colonies were scored 14 days after plating.

CAFC assays were performed by plating 2000 CB-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 kept at 37 °C/5% CO₂ for 24 h, after which CD34⁺ preincubated primary-leukemic cells were added in 100 μ L 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 μ M 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.

Drug combination analysis

Drug interaction evaluation was assessed employing the combination index (CI) equation of Chou and Talalay [19] and Berenbaum [20]: $CI = (D_1/Dx_1) + (D_2/Dx_2)$. A CI value equal to one indicates additivity, values less than one indicate synergy, and values greater than one indicate antagonism. 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: $\text{Dose (x)} = \text{GI}_{50} \times (\text{max} - \text{response}/\text{response} - \text{min})^{1/\text{hillslope}}$. GI_{50} and values for minimum (min), maximum (max) and hill slope were obtained using GraphPad Prism version 6.0b software.

Results

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 a panel of six human leukemia cell lines that included the P-gp overexpressing cell lines KG-1a (acute myeloid leukemia, AML) [21,22] and CCRF-CEM/VBL (acute lymphoid leukemia, ALL) [23] (Fig. S1). We compared their effect against daunorubicin and cytarabine clinically used chemotherapy drugs for leukemia treatment. 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 72 h and measured viability by a metabolic dye reduction assay. We found that both compounds reduced leukemia cell proliferation (Table 1). 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). Conversely, ZMP showed subnanomolar 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). Taken together, these results demonstrate that compounds with high affinity or covalent binding are able to circumvent the effect of pump efflux.

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 24 h, and measured cell cycling by DNA deconvolution. We found that ZMP and CTX-40 induced cell cycle arrest characterized by an increase of the fraction of cells in G_2/M phase and a decrease of the cell fraction at G_0/G_1 (Fig. 2). The cycle arrest in MV4-11 occurred at 12 h (Fig. 2A), while in the P-gp overexpressing cell line KG-1a the effect was more evident at 24 h (Fig. 2B). To determine whether the G_2/M arrest was followed by cell death we analyzed the induction of apoptosis by determining the activation of caspase-3 and -7. We found that both compounds induced activation of caspase-3/-7 (Fig. 3). In the presence of caspase-8 or -9 inhibitors this activity was notably reduced (Fig. 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.

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

To determine whether ZMP and CTX-40 synergize with daunorubicine we exposed chemoresistant (KG-1a) and chemosensitive (MV4-11) AML cells to the concurrent and sequential combination of these drugs to measure their anti-proliferative effect. The combinatorial effect was determined by the combination index (CI) [19,20], where CI values less than 1.0 indicate 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 2). Remarkably, these results indicated a statistically significant effect for the combination even when ZMP and CTX-40 were administered at concentrations four times lower than their GI_{50} (Table 2). There were no changes in the synergistic effect when drugs were administered sequentially (i.e. daunorubicin for 24 h followed by ZMP or CTX-40) (Table S1). Notably, the combination of ZMP and CTX-40 with daunorubicin was synergistic even in the chemoresistant AML cell line KG-1a, suggesting a potential way to circumvent anthracycline resistance.

***In vitro* toxicity of ZMP and CTX-40 toward CD34⁺ normal hematopoietic cells is equivalent to cytarabine**

To determine the effect of ZMP and CTX-40 on human normal hematopoietic stem cells (HSCs) 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, HSCs are recognized *ex vivo* via the formation of the so called “cobblestone areas” (the burrowing of HSCs beneath a monolayer of bone marrow fibroblasts that results in the formation of phase-contrast dark areas of tightly associated cells) [24]. 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 was observed with the cytarabine pretreatment (Fig. 4A left). All 3 compounds induced a higher acute inhibition of the erythroid lineage compared to the granulocyte/macrophage lineage. Lower doses (0.15 and 0.3 nM) of CTX-40 induced a slightly higher inhibitory effect than ZMP, however both compounds exhibited similar activities at higher concentrations (0.6 and 1.2 nM). We 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) (Fig. 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 progenitor cells were found to be 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 effects to cytarabine in all tested concentrations (Fig. 4B left). The cobblestones observed in the pretreated samples with cytarabine, ZMP or CTX-40 presented equivalent morphologies to those observed in the control group (Fig. 4B right).

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 for 72 h with ZMP or CTX-40 (vs. vehicle) and after drug washout were 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 AML cell lines employed in our study, showing GI_{50} of 0.3 and 0.8 nM for ZMP and CTX-40, respectively. ZMP proved to be slightly more effective than CTX-40 against the leukemic stem cell fraction (Fig. 5A and B).

Discussion

In the present study we have demonstrated that microtubule-stabilizing agents (MSA) with high binding affinity or covalent binding could effectively avoid pump-efflux from AML cells, a major cause of treatment failure in these patients. Our results demonstrate that the marine MSA 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 anti-leukemic 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-fold more potent than cytarabine. We obtained a resistance ratio, i.e. $GI_{50}(\text{resistant})/GI_{50}(\text{sensitive})$, in AML cell lines of around 650 for paclitaxel and 13 for CTX-40, making CTX-40 approximately 50-times more active than paclitaxel. Collectively, our results indicate that CTX-40 is less affected by P-gp-mediated resistance than its parental compound paclitaxel.

Although ZMP and CTX-40 decreased the number of normal hematopoietic 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 is found in 30% of all AML cases [25] and is associated with an aggressive disease phenotype and poor outcome [26]. 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 have demonstrated that it 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to acknowledge all Moore lab members for insightful discussions and Ms. Katharine Debeer for helping in the manuscript preparation. L.C. is the Weill Cornell Raymond and Beverly Sackler Scholar. This work was supported by the Sackler Foundation (L.C.), the Hirsch Fund (L.C.) and the NSFC (Grant No. 30930108).

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2015.07.038.

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

Fig. 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 h. The abundance of cells in each cell cycle phase is represented as a percentage of the total.

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

Fig. 4.

In vitro toxicity of zampanolide (ZMP) and CTX-40 on hematopoietic progenitor and stem cells. (A) Colony formation of 1000 pulse-treated (72 h) CB-CD34⁺ stem/progenitor cells with cytarabine (Ara-C), ZMP or CTX-40. On the right, images of the observed colonies at week 2. (B) The effects of Ara-C, ZMP or CTX-40 on cobblestone formation of CB-CD34⁺ cells. Right, representative images of the observed cobblestones at week 5.

Fig. 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).

Table 1

Cell growth inhibition 50 GI₅₀ at 72 h in nM by paclitaxel (PTX), daunorubicin (DAU), cytarabine (Ara-C), zampanolide (ZMP) and CTX-40 against a panel of human leukemic cell lines as determined by Alamarblue assay. The results are expressed as $\pm$ mean SEM from at least three independent experiments. effect indicates the ratio of the GI₅₀ of the resistant lines with respect to the GI₅₀-mean obtained in the sensitive lines. AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia.

Histology	Cell line	GI ₅₀ (nM)		effect (ZMP)						
		DAU	effect (DAU)	Ara-C	effect (Ara-C)	PTX	effect (PTX)	CTX-40	effect (CTX-40)	ZMP
AML	MV4-11	10.8 (±2.6)	×10.2	36 (±10)	×1.4	2.9 (±0.3)	×58.4	0.3 (±0.04)	×1.4	0.22 (±0.03)
	HL-60	12.3 (±3.5)	<i>p</i> = 0.0002	14.9 (±0.5)	<i>p</i> = 0.1703	2.2 (±0.4)	<i>p</i> = 0.0011	0.43 (±0.1)	<i>p</i> = 0.0158	0.35 (±0.05)
ALL	KG-1a	118 (±27)		36 (±5)		149 (±59)		0.5 (±0.05)		0.22 (±0.03)
	Reh	4.3 (±1.4)	×61.3	32 (±8)	×0.3	4.2 (±0.3)	×505	0.3 (±0.03)	×17.5	0.3 (±0.05)
	CCRF-CEM	24.4 (±2.1)	<i>p</i> = 0.0167	12.7 (±3)	<i>p</i> = 0.4242	3.1 (±0.3)	<i>p</i> = 0.0167	0.58 (±0.06)	<i>p</i> = 0.0040	0.43 (±0.03)
	CCRF-CEM/VBL	880 (±321)		13.2 (±1.5)		1,844 (±61)		7.7 (±0.9)		0.7 (±0.1)

Table 2

Combination Index (CI) values for ZMP and CTX-40 combinations with DAU in KG1-a and MV4-11 cells.

KG1-a		MV4-11										
DAU (nM)	ZMP (nM)	CTX-40 (nM)	CI $\pm$ SEM	n	P value	DAU (nM)	ZMP (nM)	CTX-40 (nM)	CI $\pm$ SEM	n	P value	
118	0.22	–	7.01 $\pm$ 2.00	5	0.0397	10.8	0.22	–	2.09 $\pm$ 0.92	5	ns	
59	0.11	–	1.54 $\pm$ 0.26	5	ns	5.4	0.11	–	0.87 $\pm$ 0.39	5	ns	
29.5	0.06	–	0.51 $\pm$ 0.08	5	0.0044	2.7	0.06	–	0.21 $\pm$ 0.09	5	0.0010	
14.75	0.03	–	0.27 $\pm$ 0.04	5	<0.0001	1.35	0.03	–	0.08 $\pm$ 0.03	4	0.0001	
7.38	0.01	–	0.08 $\pm$ 0.01	4	<0.0001	0.68	0.01	–	0.02 $\pm$ 0.01	5	<0.0001	
118	–	0.25	4.95 $\pm$ 2.03	4	ns	10.8	–	0.3	1.62 $\pm$ 0.40	5	ns	
59	–	0.13	1.31 $\pm$ 0.49	4	ns	5.4	–	0.15	0.61 $\pm$ 0.13	5	ns	
29.5	–	0.06	0.42 $\pm$ 0.07	4	0.0032	2.7	–	0.08	0.23 $\pm$ 0.05	5	<0.0001	
14.75	–	0.03	0.21 $\pm$ 0.05	4	0.0007	1.35	–	0.04	0.10 $\pm$ 0.01	4	<0.0001	
7.38	–	0.02	0.06 $\pm$ 0.01	3	0.0001	0.68	–	0.02	0.02 $\pm$ 0.01	3	<0.0001	

Calculated values for the combination index (CI) are presented for daunorubicin (DAU) paired with zampanolide (ZMP) or CTX-40 in KG-1a and MV4-11 cell lines. Drugs were added at the same time (concurrency). Concentrations are given in nanomolar. P values are calculated from a one-sample Student's test, and the number of biological replicates (n) is given. CI values showing significant synergistic interactions are presented in bold.