scientific reports

Check for updates

OPEN

(*R*)-WAC-224, a new anticancer quinolone, combined with venetoclax and azacitidine overcomes venetoclax-resistant AML through MCL-1 downregulation

Hiroshi Ureshino[1]✉, Taichi Ueshima[2], Tomonori Yamaguchi[2], Miyuki Takashima[2], Yusuke Sanuki[2] & Tatsuo Ichinohe[1]

Hypomethylating agents combined with venetoclax (VEN), a BCL-2 inhibitor, represent a standard treatment strategy for patients with acute myeloid leukemia (AML). Although this combination is highly effective, acquired resistance commonly occurs. MCL-1, a BCL-2 family molecule, is frequently upregulated in VEN-resistant cells, playing a major role in VEN resistance. Previously, we demonstrated that (*R*)-WAC-224 is effective against AML with minimal cardiac toxicity. (*R*)-WAC-224 combined with VEN demonstrated strong antileukemia effects on VEN-resistant AML cells overexpressing MCL-1 in vitro. Gene expression profiles revealed that (*R*)-WAC-224 with VEN induced DNA damage pathways leading to cell apoptosis. (*R*)-WAC-224 elicited caspase 3 activation, which cleaved MCL-1; this effect was reversed by a caspase inhibitor, thus overcoming VEN resistance. A combination of azacitidine (AZA), a hypomethylating agent, VEN, and (*R*)-WAC-224 was highly effective against VEN-resistant AML in vivo without increasing toxicity. (*R*)-WAC-224 exhibited antileukemia effects on VEN-resistant AML via MCL-1 downregulation in vitro and in vivo. The combination of AZA, VEN, and (*R*)-WAC-224 may be a promising treatment strategy for patients with AML.

Keywords Acute myeloid leukemia, (*R*)-WAC-224, Anticancer quinolone, Venetoclax, MCL-1

Acute myeloid leukemia (AML) develops from clonal hematopoietic stem and progenitor cells through the accumulation of genetic and epigenetic aberrations[1]. The combination of venetoclax (VEN), a selective BCL-2 inhibitor, and azacitidine (AZA), a hypomethylating agent, has become a standard chemotherapy regimen for newly diagnosed AML patients ineligible for conventional chemotherapy and/or allogeneic stem cell transplantation due to its high response rates and favorable safety profile[2]. However, patients with AML who experience relapse or become refractory to VEN and AZA combination therapy face extremely poor outcomes. Consequently, primary and/or acquired resistance to this combination therapy presents a significant obstacle to achieving long-term remission in AML patients[3]. Treatment with VEN can lead to the development of resistance through the upregulation of alternative antiapoptotic pathways. MCL-1, a member of the BCL-2 family, is frequently upregulated in VEN-resistant cells, thus playing a major role in the VEN-resistance mechanism[4,5] Targeting MCL-1 may potentially overcome VEN resistance[6].

We developed a novel anticancer quinolone, 5-methyl-7-[3-({[(2R)-oxan-2-yl]methyl}carbamoyl)azetidin-1-yl]-4-oxo-1-(1,3-thiazol-2-yl)-1,4-dihydro-1,8-naphthyridine-3-carboxylic acid [*(R)*-WAC-224 (*R*-WAC)]. *(R)*-WAC-224 exhibited superior anti AML efficacy by directly inhibiting topoisomerase-2, compared to anthracycline, with favorable safety profiles in both monotherapy and combination with cytarabine[7]. This study evaluated the efficacy and safety of *(R)*-WAC-224 as monotherapy and in combination with AZA and VEN in AML to determine whether the addition of *(R)*-WAC-224 could overcome VEN resistance.

[1]Department of Hematology and Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University, 1-2-3, Kasumi, Minami-ku, Hiroshima 734-8553, Japan. [2]Drug Discovery Laboratory, Wakunaga Pharmaceutical Co., Ltd., Hiroshima, Japan. ✉email: hureshin@hiroshima-u.ac.jp

Material and method

Reagents

5-methyl-7-[3-({[(2R)-oxan-2-yl]methyl}carbamoyl)azetidin-1-yl]-4-oxo-1-(1,3-thiazol-2-yl)-1,4-dihydro-1,8-naphthyridine-3-carboxylic acid [*(R)*-WAC-224 (R-WAC)], was synthesized by Wakunaga Pharmaceutical (Hiroshima, Japan, Fig. 1). Azacitidine (AZA) and MG132 (proteasome inhibitor) was obtained from FUJIFILM Wako Pure Chemical Corporation (Tokyo, Japan), venetoclax (VEN) from Enamine (Kyiv, Ukraine) and Z-VAD-FMK (pan-caspase inhibitor) from PEPTIDE INSTITUTE, Inc. (Osaka, Japan). All reagents used for in vitro studies were dissolved in dimethyl sulfoxide (DMSO) and stored at – 20 °C.

Cell lines and cultures

HEL92.1.7, KG-1, HL-60, TF-1, MV4-11, and MOLM13 cell lines were obtained from the American Type Culture Collection (MD, USA). HEL92.1.7, HL-60, and MOLM13 were cultured in RPMI1640 medium *(*FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan*)* supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, MA, USA) and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin, Thermo Fisher Scientific). TF-1 cells were maintained in RPMI1640 medium containing 10% FBS, antibiotics, and 2 ng/mL GM-CSF (Biolegend, CA, USA). KG-1 cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM, Thermo Fisher Scientific) supplemented with 20% FBS and antibiotics. MV4-11 cells were maintained in IMDM supplemented with 10% FBS and antibiotics. All cell lines were used within 2 months of thawing or within 25 passages.

VEN-resistant cell lines

To establish VEN-resistant AML cell lines (HEL92.1.7R, KG-1R, HL-60R, TF-1R, MV4-11R, and MOLM13R), each cell line was cultured with low concentrations of VEN, gradually increasing the concentration step by step over approximately three months. Before use in evaluations, the established VEN-resistant cell lines were tested for their sensitivity to VEN to confirm that resistance was sustained. The concentrations of VEN used for cell maintenance are as follows: HEL92.1.7 (40 µM), KG-1(40 µM), HL-60 (20 µM), TF-1 (40 µM), MV4-11 (20 µM) and MOLM13 (10 µM).

Cell growth assay

Cell proliferation was assessed after 72 h of incubation with the compounds using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technology, Kumamoto, Japan). Half-maximal inhibitory concentration (IC_{50}) values were calculated using Kyplot 6.0 (KyensLab inc, Tokyo, Japan). The combination index (CI) was calculated using the IC_{50} values of Drug A alone, Drug B alone, and the combination of Drug A and B, as described below (Chau-Talalay method)[8].

$$\mathrm{CI} = \frac{a}{A} + \frac{b}{B}$$

A: Drug A alone, B: Drug B alone, a: Drug A combination, b: Drug B combination.

Apoptosis assays

MV4-11, MV4-11R, KG-1, KG1R, HL60, and HL60R cells were seeded at a concentration of 2.5×10^5 cells/mL and incubated for 24 h with vehicle, (*R*)-WAC-224 (1.0 µM for MV4-11, KG1 and HL60, 3.0 µM for HL60R, 10 µM for MV4-11R and KG1R), VEN (30 nM for MV4-11, 100 nM for MV4-11R and HL60, 300 nM for KG-1, 10 µM for HL60R, 30 µM for KG1R), or (*R*)-WAC-224 plus VEN. Cells were harvested and washed with

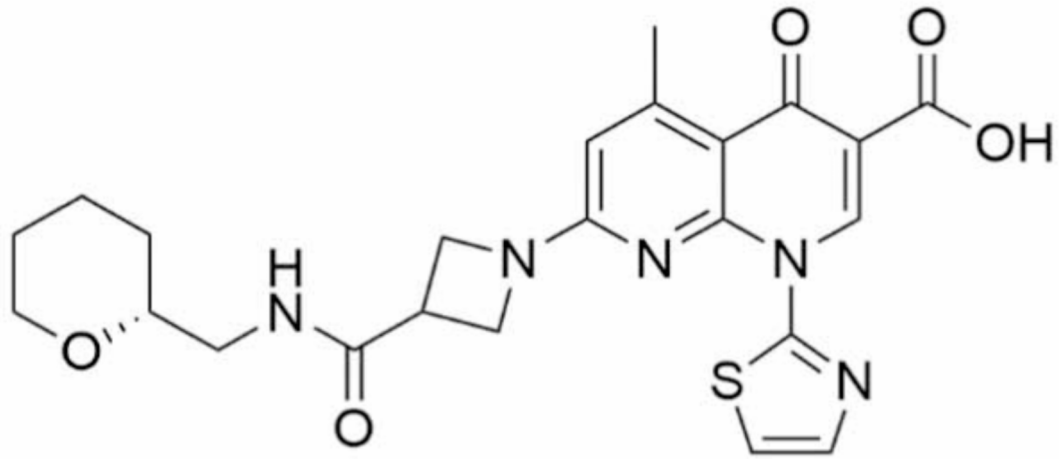

Fig. 1. Structure of (*R*)-WAC-224.

phosphate-buffered saline. Subsequently, cells were stained using the MEBCYTO Apoptosis Kit (MEDICAL & BIOLOGICAL LABORATORIES CO., LTD, Tokyo, Japan) following the manufacturer's instructions. Stained cells (Annexin V-FITC and Propidium Iodide) were analyzed using CytoFLEX Flow cytometers (Beckman Coulter, Tokyo, Japan).

Western blot analysis

Whole-cell lysates were extracted from cell lines treated with the indicated compounds using RIPA buffer (Nacalai Tesque, Kyoto, Japan). The buffer contained a Halt protease and phosphatase inhibitor cocktail (ThermoFisher Scientific). Protein concentrations were determined using the BCA protein assay kit (TakaraBio, Shiga, Japan). Equal amounts of whole-cell lysate were denatured in SDS sample buffer for 5 min at 98 °C. The sample buffer composition was 75 mM Tris–HCl (pH 6.8), 12% SDS, 600 mM DTT, 30% glycerol, and 0.03% bromophenol blue. Denatured samples were separated on 10–20% e-PAGEL gel (ATTO, Tokyo, Japan). The separated proteins were then transferred to Immobilon-Psq PVDF membranes (Millipore, MA, USA). Immunoblotted bands were detected using the HRP detection reagent (Millipore). The following primary and secondary antibodies were used: monoclonal anti-MCL-1 (Cell Signaling Technology, MA, USA), polyclonal anti-MCL-1 (Proteintech, IL, USA and Cell Signaling Technology), anti-BCL-xL (Proteintech), anti-PARP, anti-Cleaved Caspase-3 (Cell Signaling Technology, MA, USA), monoclonal anti-β-actin (Cell Signaling Technology), anti-rabbit IgG HRP-linked antibody, and anti-mouse IgG HRP-linked antibody (Cell Signaling Technology).

RNA sequencing (RNA-seq)

Total RNA was isolated from MV4-11 cells treated with vehicle (DMSO; control), 0.5 μM (*R*)-WAC-224, 0.01 μM VEN, or a combination of 0.5 μM (*R*)-WAC-224 and 0.01 μM VEN for 24 h using the Direct-zol RNA Microprep kit (Zymo Research, CA, USA). RNA quantity and quality were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific). For the MGI platform, RNA-seq libraries were prepared from total RNA using the Optimal Dual-Mode mRNA Library Prep Kit (MGI Genomics, Wuhan, China) following the manufacturer's protocol. Single-end 100-cycle sequencing runs were performed on the MGI DNBSEQ G400 using the DNBSEQ-G400RS High-Throughput Sequencing Kit (MGI, Wuhan, China). RNA extraction, mRNA isolation, library preparation, sequencing, and analysis were performed by BGI Genomics (Wuhan, China).

Xenograft mouse models

MV4-11 cells (1×10^7 cells/mouse) were subcutaneously inoculated into the right dorsal site of 6-week-old female Balb/c-nu/nu mice (Japan SLC, Inc., Shizuoka, Japan). Eleven days post-transplantation, mice were randomized into eight groups to ensure equal tumor sizes across groups (vehicle, 15 mg/kg of (*R*)-WAC-224, 50 mg/kg of VEN, 3 mg/kg of AZA, 15 mg/kg of (*R*)-WAC-224 + 50 mg/kg of VEN, 15 mg/kg of (*R*)-WAC-224 + 3 mg/kg of AZA, 50 mg/kg of VEN + 3 mg/kg of AZA and 15 mg/kg of (*R*)-WAC-224 + 50 mg/kg of VEN + 3 mg/kg of AZA). Similarly, MV4-11R cells (1×10^7 cells/mouse) were subcutaneously inoculated into the right dorsal site of 6-week-old female Balb/c-nu/nu mice. Ten days post-transplantation, these mice were randomized into seven groups (vehicle, 50 mg/kg of VEN, 50 mg/kg of VEN + 3 mg/kg of AZA, 15 mg/kg of (*R*)-WAC-224, 20 mg/kg of (*R*)-WAC-224, 15 mg/kg of (*R*)-WAC-224 + 50 mg/kg of VEN + 3 mg/kg of AZA and 20 mg/kg of (*R*)-WAC-224 + 50 mg/kg of VEN + 3 mg/kg of AZA). Treatment commenced on day 0 and continued until day 14. (*R*)-WAC-224 or its vehicle (10 mM NaOH plus 5% glucose) was administered intravenously once every seven days (days 0, 7, and 14). VEN or its vehicle (60% Polyoxyethylene (10) Hydrogenated Castor Oil, 35% Polyethylene glycol 400, and 5% Ethanol) was administered orally for five consecutive days followed by two days without administration (days 0–4, 7–11, 14). AZA or its vehicle (saline) was injected intraperitoneally every other day or every two days (days 0, 2, 4, 7, 9, 11, 14). Tumor volumes, calculated as $(\text{short axis})^2 \times (\text{long axis})/2$, were measured twice weekly. All mice were euthanized by exsanguination under anesthesia with 4% isoflurane on day 14. These experiments were conducted in accordance with protocols approved by the Wakunaga Pharmaceutical Company Institutional Animal Care and Use Committee (Permission No. 375) and conform to the Guide for the Care and Use of Laboratory Animals published by the United States National Institute of Health. The study adhered to ARRIVE guidelines.

Statistical analysis

All data are expressed as mean ± standard deviation. One-way ANOVA followed by Tukey–Kramer test determined significant differences when comparing three or more groups, while t-test was applied for comparisons between two groups. *P*-values < 0.05 were considered statistically significant. Statistical analyses were performed using Kyplot 6.0 (KyensLab Incorporated, Tokyo, Japan) or FreeJSTAT Version 22.0E (M. Sato, Japan).

Results

VEN-resistant AML cells exhibited upregulated MCL-1 levels

We established VEN-resistant AML cell lines (HEL92.1.7R, KG-1R, HL-60R, TF-1R, MV4-11R, and MOLM13R). The IC_{50} values of these resistant lines were significantly higher than those of their respective parental cell lines (Fig. 2A, Table 1). Although the precise frequency of MCL-1 overexpression among VEN-resistant patients remains unclear, evidence from patient-derived xenograft models suggests that MCL-1 upregulation represents one of the key mechanisms underlying VEN resistance in AML[9–11]; thus, we assessed MCL-1 and other BCL2 family proteins, BCL-xL and BCL-2 expression levels in the VEN-resistant cell lines. Western blot analysis revealed elevated MCL-1 protein levels in VEN-resistant cells (MV4-11, HL-60 and MOLM13) compared to their parental cell lines, consistent with previous reports (Fig. 2B)[9–11]. For other BCL-2 family proteins, an upregulation of BCL-2 and BCL-xL expression was observed in HL60 cells. However, in MV4-11 and MOLM13 cells, no consistent changes in BCL-xL and BCL-2 expression were observed between parental and resistant cells.

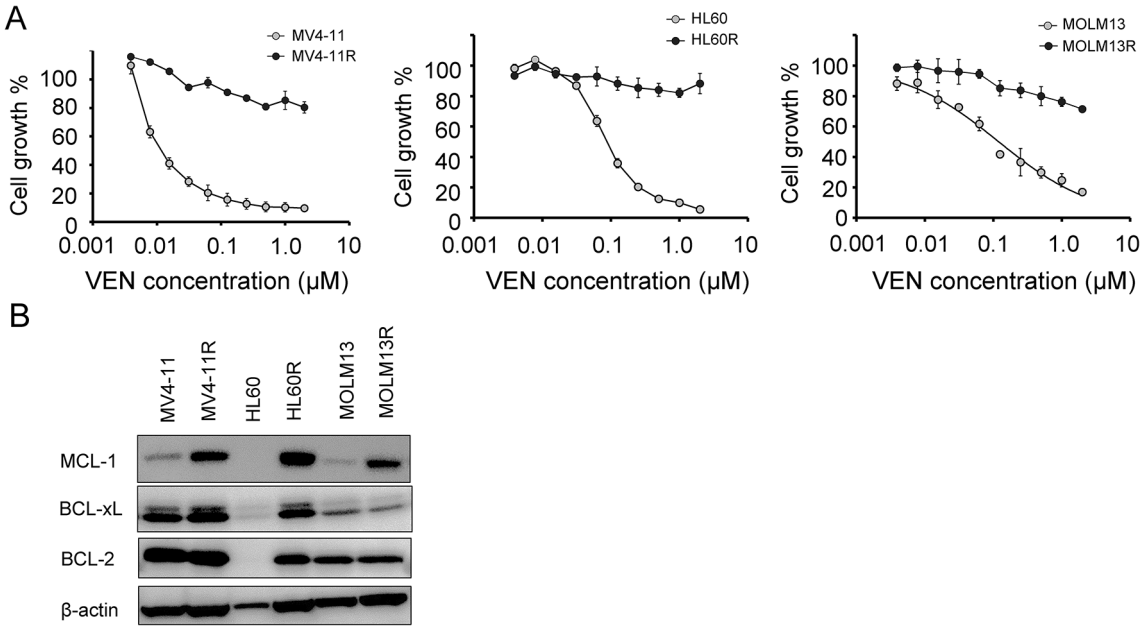

Fig. 2. Features of venetoclax (VEN) resistant cell lines. (**A**) Cell growth inhibition was assessed in parental and VEN-resistant MV4-11, HL-60 and MOLM13 cell lines. (**B**) MCL-1, BCL-xL and BCL-2 protein levels in parental and VEN-resistant cell lines (MV4-11, HL-60 and MOLM13 cells).

		HEL92.1.7	KG-1	HL60	TF-1	MV4-11	MOLM-13
Parent cell lines	VEN	4.79	1.15	0.02	2.61	0.02	0.01
	AZA	10.04	21.65	3.96	13.63	2.78	2.59
	WAC	0.14	1.00	0.08	3.16	0.02	0.02
	VEN-R	**HEL92.1.7**	**KG-1**	**HL60**	**TF-1**	**MV4-11**	**MOLM-13**
VEN-resistant cell lines	VEN	12.48	25.95	8.35	16.56	5.10	3.80
	AZA	8.07	17.45	3.86	52.67	2.84	2.80
	WAC	0.18	1.07	0.09	4.74	0.15	0.02

Table 1. IC_{50} of venetoclax (VEN), azacitidine (AZA) and (*R*)-WAC-224 (WAC) in acute myeloid leukemia cell lines (μM).

(*R*)-WAC-224 demonstrated cell growth inhibition against VEN-resistant AML cells

We investigated the cell growth inhibition effects of (*R*)-WAC-224 on VEN-resistant AML. (*R*)-WAC-224 and azacitidine (AZA), a common combination partner of VEN, demonstrated comparable cell growth inhibition in both parental AML cells and VEN-resistant cells (Table 1). Subsequently, we evaluated the combined effects of (*R*)-WAC-224 and VEN. The combination of (*R*)-WAC-224 and VEN exhibited greater cell growth inhibition compared to each monotherapy in parental MV4-11, HL60 and MOLM13 cells. Notably, this combination also demonstrated efficacy in several VEN-resistant cells. The combination index was below 1.0 in both parental and resistant cells of MV4-11, HL60, and MOLM13, indicating a synergistic effect. These findings suggest that (*R*)-WAC-224 may overcome VEN resistance in AML (Fig. 3A).

(*R*)-WAC-224 reduced MCL-1 expression in parental and VEN-resistant AML cells

We investigated the mechanisms by which (*R*)-WAC-224 overcomes VEN resistance in AML, focusing on MCL-1. Treatment with (*R*)-WAC-224 downregulated MCL-1 protein levels in parental and VEN-resistant AML cells in a dose-dependent manner (Fig. 3B). Co-exposure of MV4-11 to (*R*)-WAC-224 and the proteasome inhibitor, MG-132 (5 μM) prevented MCL-1 downregulation (Fig. 3C), indicating proteasomal degradation.

The combination of (*R*)-WAC-224 and VEN strongly induced cell apoptosis through DNA damage

Having shown that (*R*)-WAC-224 plus VEN exhibited strong cell growth inhibition against both parental and VEN-resistant MV4-11 cells, we investigated the mechanism underlying the strong cell growth inhibition observed with the combination therapy using RNA sequencing. We analyzed the gene expression profiles of MV4-11 cells following treatment with vehicle, (*R*)-WAC-224 monotherapy, VEN monotherapy, and (*R*)-WAC-224 plus VEN combination. Total RNA was extracted from cells 24 h post-treatment with vehicle or drugs.

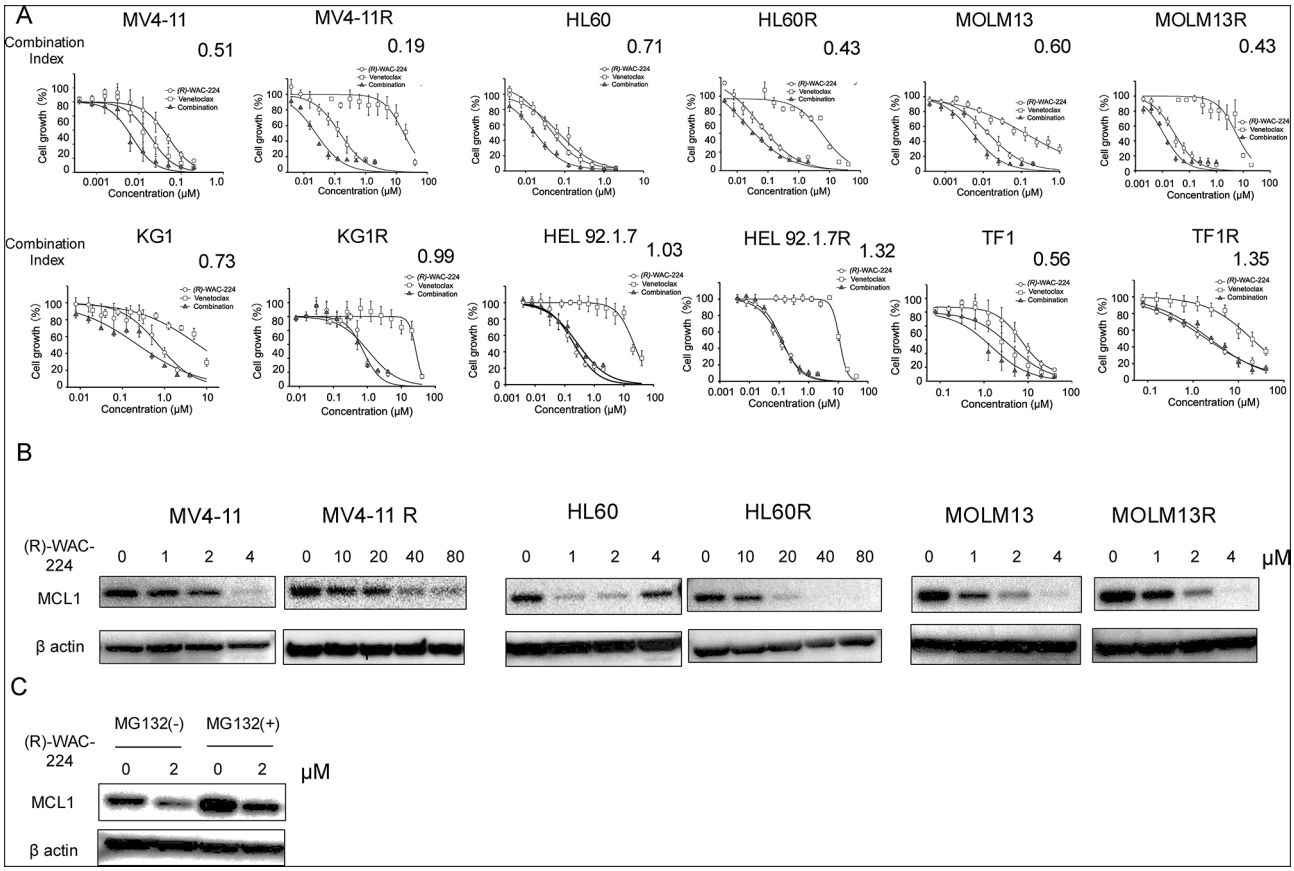

Fig. 3. Cell growth inhibition and MCL-1 protein levels in parental and venetoclax (VEN) resistant acute myeloid leukemia (AML) cell lines. (**A**) The combination of (*R*)-WAC-224 and VEN demonstrated cell growth inhibition against both parental and VEN-resistant AML cells. The combination index (CI) values showed synergistic effects in both parental and resistant cells of MV4-11, HL60, and MOLM13. (**B**) Treatment with (*R*)-WAC-224 downregulated MCL-1 protein levels in parental and VEN-resistant MV4-11, HL60 and MOLM13 cell lines in a dose-dependent manner. (**C**) Effect of MG132 on MCL1 expression in MV4-11 cells treated with (*R*)-WAC-224.

Our analysis revealed that 1,184, 29, and 1696 genes were differentially expressed in MV4-11 cells following treatment with (*R*)-WAC-224 monotherapy, VEN monotherapy, and (*R*)-WAC-224 plus VEN combination, respectively. Differential gene expression (DEG) analysis of MV4-11 cells showed that both (*R*)-WAC-224 monotherapy and (*R*)-WAC-224 plus VEN significantly upregulated pathways related to cell cycle, DNA replication, p53 signaling, Fanconi anemia, homologous recombination, mismatch repair, and base excision repair (Significant genes; fold change ≥ 2.0, $P < 0.05$) compared to control (Fig. 4A)[12]. Further analysis focusing on apoptosis and DNA damage pathways revealed that the intrinsic apoptotic signaling pathway in response to DNA damage by p53 class mediator was also upregulated in both (*R*)-WAC-224 monotherapy and (*R*)-WAC-224 plus VEN treated cells (Fig. 4B), whereas, this pathway was not upregulated in VEN-treated cells (Supplementary Fig. 1). These findings suggest that (*R*)-WAC-224 monotherapy or (*R*)-WAC-224 plus VEN induced DNA damage leading to cell apoptosis. Flow cytometric analysis confirmed that the (*R*)-WAC-224 plus VEN combination induced stronger cell apoptosis compared to each monotherapy in MV4-11, KG-1 and HL60 cells. This effect was similarly observed in VEN-resistant cell lines (MV4-11R, KG1R and HL60R) (Fig. 4C).

(*R*)-WAC-224 downregulated MCL-1 levels via the cleaved caspase-3 pathway in parental and VEN-resistant AML cells

(*R*)-WAC-224 in combination with VEN demonstrated potent cell apoptosis induction effects. Given that MCL-1 is reportedly cleaved by caspase 3, we examined MCL-1 and cleaved caspase 3 levels in MV4-11 cells following (*R*)-WAC-224 treatment. MCL-1 levels decreased in a dose-dependent manner, corresponding to increased cleaved caspase 3 levels in MV4-11 cells. Cleaved PARP levels also decreased, while BCL-xL levels remained unchanged (Fig. 5). These effects were nullified by Z-VAD-FMK (a pan-caspase inhibitor), suggesting that MCL-1 downregulation was mediated via cleavage by caspase 3 (Fig. 5). Similar effects were observed following AZA treatment (Supplementary Fig. 2), indicating that AZA could also overcome VEN resistance through MCL-1 downregulation. Consequently, the combination of VEN with AZA plus (*R*)-WAC-224 may represent a promising treatment strategy for VEN-resistant AML.

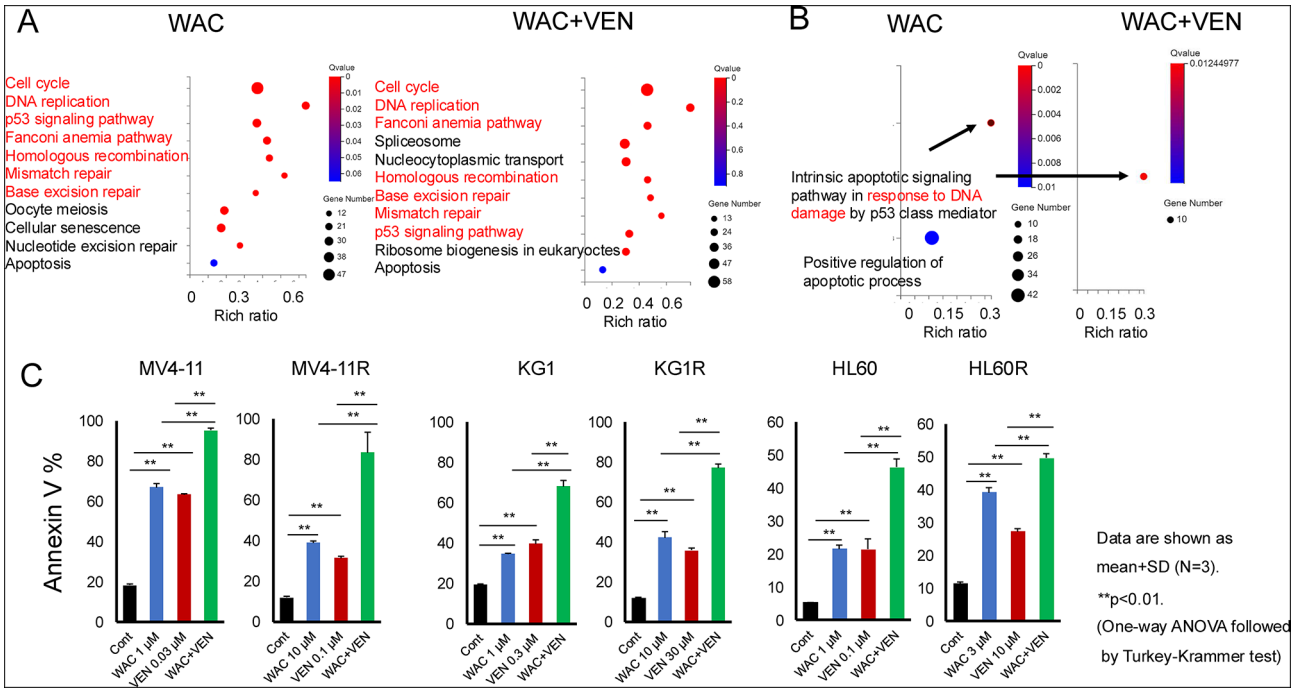

Fig. 4. (**A**) Differential gene expression analysis of MV4-11 cells revealed that (*R*)-WAC 224 (WAC) alone or (*R*)-WAC in combination with venetoclax (VEN) significantly altered cell cycle, DNA replication, and p53 signaling pathways (fold change ≥ 2.0, $P < 0.05$) compared to the control. (**B**) Further analysis focusing on apoptosis and DNA damage revealed that the intrinsic apoptotic signaling pathway in response to DNA damage mediated by p53 (Q value < 0.05) was also upregulated in both (*R*)-WAC and (*R*)-WAC plus VEN treatments. (**C**) Flow cytometric analysis demonstrated that (*R*)-WAC 224 and venetoclax (VEN) induced apoptosis in AML cells (MV4-11, MV4-11R, KG1, KG1R, HL60 and HL60R). Apoptotic cells were defined as Annexin V positive cells. **: $p < 0.01$.

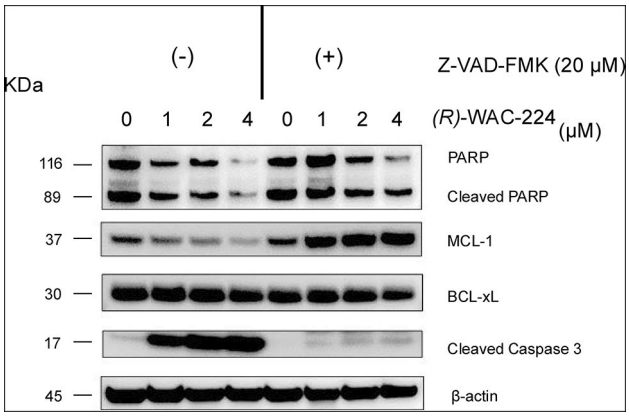

Fig. 5. Western blotting demonstrated that (*R*)-WAC-224 downregulated MCL-1 levels in a dose-dependent manner, corresponding to an increase in cleaved caspase 3. The downregulation of MCL-1 was inhibited by the pan-caspase inhibitor, Z-VAD-FMK.

(*R*)-WAC-224 in combination with AZA and VEN demonstrated antitumor efficacy in a xenograft mouse model

Finally, to examine the antileukemia effects of (*R*)-WAC-224 plus AZA and VEN in vivo, MV4-11 cells were subcutaneously inoculated into the right dorsal site of Balb/c-nu/nu mice. Eleven days post-transplantation, mice were randomized into eight groups to ensure equal tumor sizes across groups. Then, the mice were treated with vehicle, (*R*)-WAC-224, VEN, AZA, (*R*)-WAC-224 + VEN, (*R*)-WAC-224 + AZA, VEN + AZA, or (*R*)-WAC-224 + VEN + AZA for 14 days. (*R*)-WAC-224 + VEN and (*R*)-WAC-224 + VEN + AZA treatments exhibited strong antileukemia effects without increased toxicities in the MV4-11 xenografted mice (VEN + AZA, 168.4 ± 86.9 cm^3 vs. (*R*)-WAC-224 + VEN + AZA, 38.2 ± 23.9 cm^3, $p = 0.001$) (Fig. 6A,B). Subsequently, we

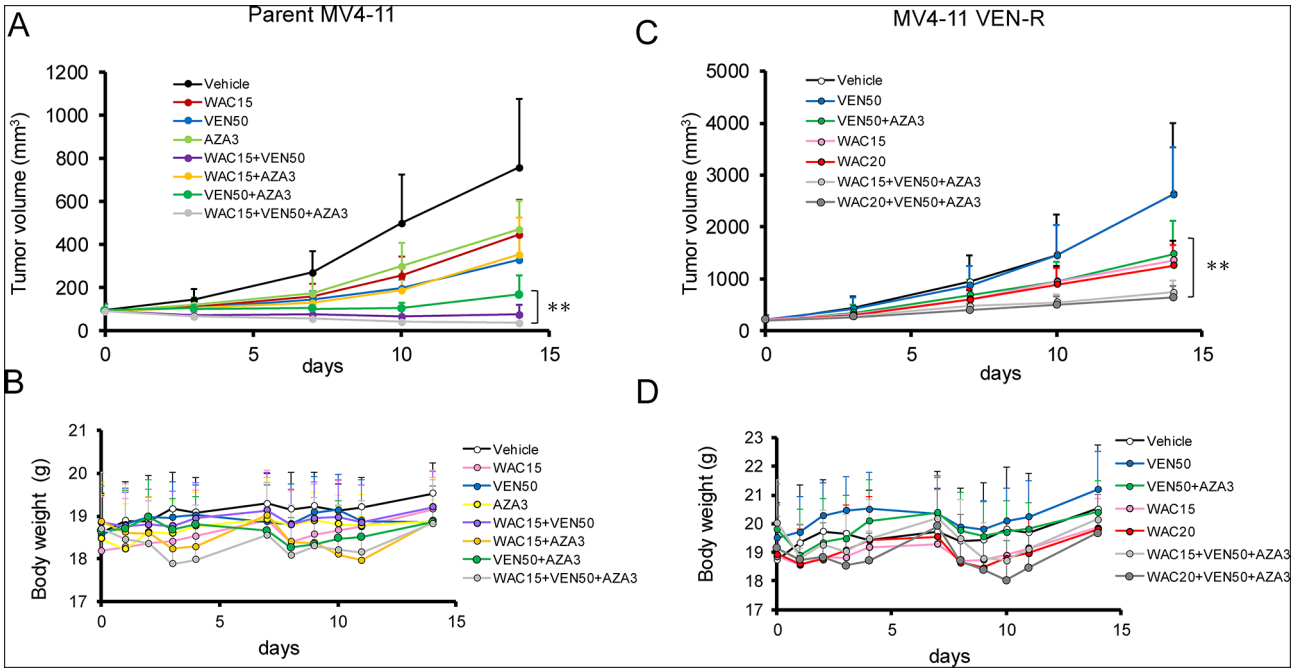

Fig. 6. Azacitidine, venetoclax, and (*R*)-WAC 224 demonstrated antitumor effects against both parental MV4-11 and venetoclax-resistant MV4-11 cells in a xenograft mouse model. Mice treated with the combination of azacitidine, venetoclax, and (*R*)-WAC 224 exhibited enhanced antileukemia effects compared to those treated with azacitidine and venetoclax alone, without increased toxicity (VEN + AZA, 168.4 ± 86.9 cm^3 vs. (*R*)-WAC-224 + VEN + AZA, 38.2 ± 23.9 cm^3, $p = 0.001$) (**A**). The triple combination treatment did not induce severe body weight loss (**B**). At a dose of 15 mg/kg, (*R*)-WAC 224 in combination with azacitidine and venetoclax demonstrated superior antileukemia effects compared to azacitidine and venetoclax alone (VEN + AZA, 1478.2 ± 641.8 cm^3 vs. (*R*)-WAC-224 (15 mg/kg) + VEN + AZA, 743.7 ± 227.4 cm^3, $p = 0.008$) (**C**). Again, mice receiving the triple combination therapy did not exhibit severe body weight loss (**D**).

investigated the antileukemia effects of (*R*)-WAC-224 plus AZA and VEN on VEN-resistant AML in vivo. MV4-11R cells were subcutaneously inoculated into the right dorsal site of 6-week-old female Balb/c-nu/nu mice. Ten days post-transplantation, mice were randomized into seven groups (vehicle, VEN, VEN + AZA, 15 mg/kg of (*R*)-WAC-224, 20 mg/kg of (*R*)-WAC-224, 15 mg/kg of (*R*)-WAC-224 + VEN + AZA, and 20 mg/kg of (*R*)-WAC-224 + VEN + AZA). The mice were treated with each drug regimen from day 0 to day 14. Both 15 mg/kg and 20 mg/kg of (*R*)-WAC-224 + VEN + AZA treatments demonstrated strong antileukemia effects without increased toxicities in the MV4-11 xenografted mice (VEN + AZA, 1478.2 ± 641.8 cm^3 vs. (*R*)-WAC-224 (15 mg/kg) + VEN + AZA, 743.7 ± 227.4 cm^3, $p = 0.008$) (Fig. 6C,D). These results suggest that (*R*)-WAC-224 + VEN + AZA might be a promising treatment strategy for VEN-resistant AML.

Discussion

We demonstrated the antileukemic effects of (*R*)-WAC-224 in VEN-resistant AML. Our previous research established that (*R*)-WAC-224 directly inhibits topoisomerase-2 and exhibits antileukemic effects both in vitro and in vivo, with reduced cardiac and intestinal toxicities[7].

Mitochondrial apoptosis is regulated by pro- and antiapoptotic BCL-2 family proteins, with upregulation of antiapoptotic proteins conferring a survival advantage in various cancers[13]. Consequently, targeting BCL-2 with hypomethylating agents has successfully provided survival benefits in patients with AML[2]. Although this combination therapy is highly effective, acquired resistance, particularly VEN resistance, commonly occurs. Upregulation of MCL-1, a major resistance mechanism for BCL-2 inhibitors, is frequently observed in VEN-resistant AML cells[9–11], and patient with high MCL-1 expression exhibit a poorer prognosis[14]. Targeting MCL-1 has proven highly effective for AML cells with acquired resistance to VEN[5,15,16]. Although MCL-1 inhibitors are highly effective, they primarily exert a functional inhibition and enhance of MCL-1 deubiquitination, leading to MCL-1 accumulation, which may result in early resistance[17]. Therefore, the degradation of MCL-1 is crucial for its effective inhibition[18].

Proteolytic cleavage of various BCL-2 family proteins by caspases has been observed in the regulation of death and survival signals. Notably, MCL-1 can be cleaved by caspase-3 in response to chemotherapy, strongly suggesting that this proteolytic cleavage might serve as a mechanism in the regulation of cell apoptosis[19]. In the present study, topoisomerase-2 inhibition by *(R)*-WAC-224 can initiate DNA damage responses and caspase-3 activation, leading to the induction of apoptotic and necro-apoptotic pathways[20]. Furthermore, (*R*)-WAC-224 promoted MCL-1 protein downregulation via caspase-3 activation leading to proteasomal degradation. Through these mechanisms, (*R*)-WAC-224 could overcome VEN-resistance. Given that (*R*)-WAC-224

functions as a topoisomerase-2 inhibitor[7], it shares a common anti-leukemia mechanism with anthracycline, daunorubicin, which also downregulates MCL-1[20] and induces DNA damage response[21]. Meanwhile, (*R*)-WAC-224 demonstrates superior safety with reduced cardiotoxicity and gastrointestinal toxicity compared to daunorubicin[7], indicating (*R*)-WAC-224 may be more suitable than daunorubicin for combination therapy with VEN. Indeed, the triple combination of (*R*)-WAC-224, AZA and VEN, and exhibited strong antileukemic activity in VEN-resistant AML without increasing toxicity in vivo.

The development of anti-leukemic quinolones has been driven by the need for effective therapies that circumvent common resistance mechanisms associated with conventional chemotherapeutic agents. Vosaroxin, a first-in-class quinolone-derived anticancer agent, exemplifies the advantages of this approach[22]. Unlike anthracyclines, vosaroxin is minimally metabolized due to its stable core quinolone structure, reducing the generation of free radicals, reactive oxygen species, and toxic metabolites. This minimizes off-target toxicity, particularly cardiotoxicity, which is a major limitation of traditional topoisomerase-2 inhibitors. Moreover, vosaroxin is not a substrate of P-glycoprotein, a key efflux transporter responsible for multidrug resistance in leukemia cells[23]. This property allows vosaroxin to retain its efficacy in drug-resistant leukemia. Our previous study demonstrated that (*R*)-WAC-224, like vosaroxin, exhibits topoisomerase-2 inhibitory activity and is not subject to resistance mediated by P-glycoprotein. Furthermore, (*R*)-WAC-224 exhibited potent anti-leukemic effects in combination with cytarabine[7]. The anti-leukemic quinolone was a promising agent; however, the development of therapies such as VEN and AZA or FLT-3 inhibitors[24] prevented its clinical implementation. Given its potential, the combination of (R)-WAC-224, VEN, and AZA represents a promising therapeutic strategy, and we aim to advance its evaluation in clinical trials.

This study has several limitations. First, while (R)-WAC-224 demonstrated potent anti-leukemic effects in vivo, its long-term efficacy and potential resistance mechanisms require further investigation. Second, although our study highlights the role of MCL-1 downregulation via caspase-3 activation, other potential pathways contributing to VEN resistance were not fully explored. Third, the in vivo experiments were conducted using xenograft models, which may not fully recapitulate the tumor microenvironment of AML patients. Clinical validation through patient-derived samples and clinical trials will be essential to confirm the translational potential of (R)-WAC-224 in overcoming VEN resistance in AML.

Data availability

RNA-sequencing data are available from the DNA Data Bank of Japan under accession number PRJDB 18,678.

Received: 24 October 2024; Accepted: 14 April 2025
Published online: 08 May 2025

References

1. DiNardo, C. D., Erba, H. P., Freeman, S. D. & Wei, A. H. Acute myeloid leukaemia. *Lancet* **401**, 2073–2086 (2023).
2. DiNardo, C. D. et al. Azacitidine and venetoclax in previously untreated acute myeloid leukemia. *N. Engl. J. Med.* **383**, 617–629 (2020).
3. Maiti, A. et al. Outcomes of relapsed or refractory acute myeloid leukemia after front-line hypomethylating agent and venetoclax regimens. *Haematologica* **106**, 894–898 (2021).
4. Thomalla, D. et al. Deregulation and epigenetic modification of BCL2-family genes cause resistance to venetoclax in hematologic malignancies. *Blood* **140**, 2113–2126 (2022).
5. Ramsey, H. E. et al. A novel MCL1 inhibitor combined with venetoclax rescues venetoclax-resistant acute myelogenous leukemia. *Cancer Discov.* **8**, 1566–1581 (2018).
6. Fischer, M. A. et al. Selective inhibition of MCL1 overcomes venetoclax resistance in a murine model of myelodysplastic syndromes. *Haematologica* **108**, 522–531 (2023).
7. Mino, T. et al. A novel anticancer quinolone, (R)-WAC-224, has anti-leukemia activities against acute myeloid leukemia. *Invest. New Drugs* **41**, 751–760 (2023).
8. Chou, T. C. & Talalay, P. Quantitative analysis of dose-effect relationships: The combined effects of multiple drugs or enzyme inhibitors. *Adv. Enzyme Regul.* **22**, 27–55 (1984).
9. Zhang, Q. et al. Activation of RAS/MAPK pathway confers MCL-1 mediated acquired resistance to BCL-2 inhibitor venetoclax in acute myeloid leukemia. *Signal Transduct. Target. Ther.* **7**, 51 (2022).
10. Pei, S. et al. Monocytic subclones confer resistance to venetoclax-based therapy in patients with acute myeloid leukemia. *Cancer Discov.* **10**, 536–551 (2020).
11. Bhatt, S. et al. Reduced mitochondrial apoptotic priming drives resistance to BH3 mimetics in acute myeloid leukemia. *Cancer Cell* **38**, 872-890.e6 (2020).
12. Kanehisa, M. & Goto, S. KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Res.* **28**, 27–30 (2000).
13. Letai, A. et al. Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. *Cancer Cell* **2**, 183–192 (2002).
14. Li, X. et al. Increased MCL-1 expression predicts poor prognosis and disease recurrence in acute myeloid leukemia. *Onco Targets Ther.* **12**, 3295–3304 (2019).
15. Kotschy, A. et al. The MCL1 inhibitor S63845 is tolerable and effective in diverse cancer models. *Nature* **538**, 477–482 (2016).
16. Hormi, M. et al. Pairing MCL-1 inhibition with venetoclax improves therapeutic efficiency of BH3-mimetics in AML. *Eur. J. Haematol.* **105**, 588–596 (2020).
17. Tantawy, S. I. et al. Mechanisms of MCL-1 protein stability induced by MCL-1 antagonists in B-Cell malignancies. *Clin. Cancer Res.* **29**, 446–457 (2023).
18. Hu, X. et al. Src inhibition potentiates MCL-1 antagonist activity in acute myeloid leukemia. *Signal Transduct. Target. Ther.* **10**, 50 (2025).
19. Weng, C., Li, Y., Xu, D., Shi, Y. & Tang, H. Specific cleavage of Mcl-1 by caspase-3 in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in Jurkat leukemia T cells. *J. Biol. Chem.* **280**, 10491–10500 (2005).
20. Alaaeldin, R. et al. Dual topoisomerase I/II inhibition-induced apoptosis and necro-apoptosis in cancer cells by a novel ciprofloxacin derivative via RIPK1/RIPK3/MLKL activation. *Molecules* **27**, 7993. https://doi.org/10.3390/molecules27227993 (2022).
21. Stuhldreier, F. et al. Pleiotropic effects of spongean alkaloids on mechanisms of cell death, cell cycle progression and DNA damage response (DDR) of acute myeloid leukemia (AML) cells. *Cancer Lett.* **361**, 39–48 (2015).

22. Ravandi, F. et al. Vosaroxin plus cytarabine versus placebo plus cytarabine in patients with first relapsed or refractory acute myeloid leukaemia (VALOR): A randomised, controlled, double-blind, multinational, phase 3 study. *Lancet Oncol.* **16**, 1025–1036 (2015).
23. Hawtin, R. E. et al. Voreloxin is an anticancer quinolone derivative that intercalates DNA and poisons topoisomerase II. *PLoS ONE* **5**, 1–10 (2010).
24. Erba, H. P. et al. Quizartinib plus chemotherapy in newly diagnosed patients with FLT3-internal-tandem-duplication-positive acute myeloid leukaemia (QuANTUM-First): A randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet* **401**, 1571–1583 (2023).

Acknowledgements
None.

Author contributions
HU, TU and TI made substantial contributions to study conception, design, data analysis, interpretation, and drafting of the original manuscript. TU, TY, MT, YS conducted the experiments, performed data curation, formal analysis, validation, investigation, and project administration, as well as contributed to manuscript review and editing. All authors approved the final version of the manuscript.

Funding
This study was supported by Wakunaga Pharmaceutical Co., Ltd, Hiroshima, Japan and in part by the Program of the network-type Joint Usage/Research Center for Radiation Disaster Medical Science of Hiroshima University, Nagasaki University, and Fukushima Medical University (to TI).

Declarations

Competing interests
HU reports personal fees from Novartis. TU, TY, MT, and YS are full-time employees of Wakunaga Pharmaceutical Co., Ltd. TI has received speaker honoraria from Novartis and research funding from Chugai Pharmaceutical Co. and Wakunaga Pharmaceutical Co., Ltd.

Ethical approval
The experiments were conducted in accordance with protocols approved by the Wakunaga Pharmaceutical Company Institutional Animal Care and Use Committee (Permission No. 375) and conform to the Guide for the Care and Use of Laboratory Animals published by the United States National Institute of Health. The study adhered to ARRIVE guidelines.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-98534-7.

Correspondence and requests for materials should be addressed to H.U.