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PRECLINICAL STUDIES

MICONIDINE acetate, a new selective and cytotoxic compound with synergic potential, induces cell cycle arrest and apoptosis in leukemia cells

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Summary

Plants are important sources of biologically active compounds and they provide unlimited opportunities for the discovery and development of new drug leads, including new chemotherapeutics. Miconidin acetate (**MA**) is a hydroquinone derivative isolated from *E. hiemalis*. In this study we demonstrated that **MA** was cytotoxic against acute leukemia (AL), solid tumor cells and cancer stem cells, with the strongest effect exhibited against AL. Furthermore, it was non-cytotoxic against non-tumor cells and did not cause significant hemolysis. **MA** blocks the G2/M phase and causes cytostatic effects, acting in a similar way to dexamethasone by increasing PML expression. The compound also triggered intrinsic and extrinsic apoptosis by modulating Bax, FasR and survivin expression. This led to an extensive mitochondrial damage that resulted in AIF, cytochrome c and endonuclease G release, caspase-3 and PARP cleavage and DNA fragmentation. We have further demonstrated that **MA** was strongly cytotoxic against neoplastic cells collected from patients with different AL subtypes. Interestingly, **MA** increased the cytotoxic effect of chemotherapeutics cytarabine and vincristine. This study indicates that **MA** may be a new agent for AL and highlights its potential as a new source of anticancer drugs.

Keywords Cell death · Acute leukemia · Cytotoxicity · Apoptosis · Miconidine acetate

Introduction

Plants provide unlimited opportunities for the discovery and development of new drug leads, including new chemotherapy agents [1–3]. In fact, out of the 121 drugs prescribed for cancer treatment to date, 90 are derived from plant sources [4]. Furthermore, out of the 65 new pharmaceuticals registered between 1981 and 2002 for cancer, 48 were obtained from natural products [5]. Paclitaxel (Taxol), isolated from *Taxus brevifolia* [6], and vinca alkaloids, present in *Catharanthus roseus* [7], are two of the most well-known chemotherapeutics obtained from plants. Despite being one of the areas with the greatest plant diversity in the world, Brazil had only about 10% of its flora evaluated for biological activity [8]. Therefore, it is highly likely that there will be new molecules from Brazilian plants with potential activity for the development of new chemotherapeutics. The *Myrtaceae* family is represented in Brazil by about 23 genera and one thousand species, approximately one-third of which them belong to the genus *Eugenia*. This genus is widely used in folk medicine and by native peoples to treat diseases [9, 10]. In addition, numerous studies have investigated the chemical composition and biological activities of its extracts and fractions and, due to the large amount of phenolic compounds, species of the this genus have a strong antioxidant activity [11–13].

In this context, miconidine acetate **(MA)** (Fig. 1) is a hydroquinone derivative isolated from the leaves and floral buds of *Eugenia hiemalis* Cambessèdes [14, 15]. While miconidine, a hydroquinone isolated from the genus *Miconia* (Melastomataceae) (Goijman et al., 1984) has been

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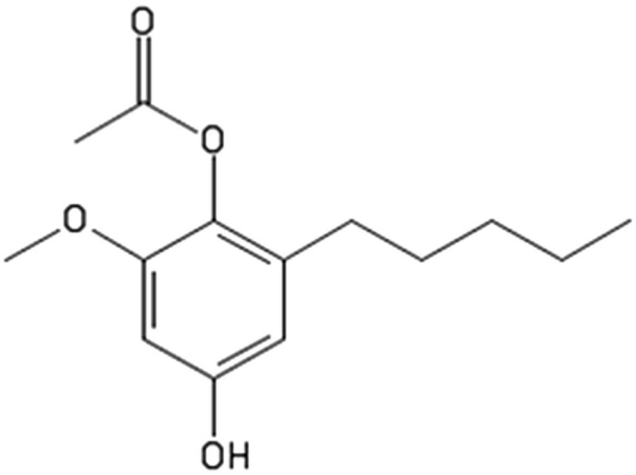

Fig. 1 Miconidine acetate (MA) chemical structure. The compound, previously isolated from the leaves and floral buds of *E. hiemalis,* was identified by spectroscopic and spectrometric techniques and also by comparison to the literature

well reported for several of its properties, including antibacterial, antimalarial, antitumor and cytostatic [14, 16–19], **MA** has not been explored by the scientific community to date. In fact, only one study [14] was found on this compound, in which it was tested in KG1a acute leukemia (AL) cells, showing some cytotoxic activity. However, the mechanisms involved in cell death induced by **MA** are still unclear.

Considering the poor prognosis of AL, the strong toxicity of currently available chemotherapeutics and the high relapse rates, most cases of this disease remain incurable, making the search for new antineoplastic compounds an urgent necessity [20, 21]. As isolated compounds from the genus *Eugenia* are promising, in this study we aimed to evaluate the effects of **MA** on different cancer cells and on non-tumor models, as well as to determine its main cytotoxic mechanisms. As **MA** was more effective against AL cells, we chose two cell lines, from myeloid and lymphoid lineages, to investigate the effects of **MA** on the cell cycle, cell proliferation, intrinsic and extrinsic apoptosis and ROS production. We also evaluated its activity on cells collected from patients with different AL subtypes and in combination with chemotherapeutics used in clinics.

Material and methods

Plant material

The aerial parts of *E. hiemalis* were collected in April 2014 in Porto Alegre (Rio Grande do Sul, Brazil) and identified by Dr. J.A. Jarenkow (Department of Botany, University of Rio Grande do Sul, Brazil). A voucher specimen was deposited in the herbarium Instituto de Ciências Naturais (University of Rio Grande do Sul) under the number ICN 127910.

Extraction and isolation

The fresh leaves (355 g) and flower buds (27 g) of *E. hiemalis* were extracted separately with dichloromethane (1.8 L and 0.25 L, respectively) at room temperature by maceration. The extracts were concentrated and reduced at 40 °C, obtaining 9.10 g and 0.49 g of crude extracts, respectively to leaves and flower buds. The crude dichloromethane leaves extract was fractionated by vacuum liquid chromatography on silica gel (40–63 μm, Sigma-Aldrich®, Missouri, USA) with hexane, chloroform, ethyl acetate and methanol, and sixteen fractions (A1-A16) were obtained. Fractions A7 and A8 were further purified by medium pressure liquid chromatography (MPLC) over silica gel (40–63 μm, Sigma-Aldrich®, Missouri, USA) using a gradient of hexane and ethyl acetate (100/0 to 0/100). The isolated compound was identified as miconidine acetate (**MA**) by spectroscopic and spectrometric techniques, including UV, IR, NMR and MS, and comparison to the literature.

Miconidin acetate (MA) Yellow oil; R_f 0.3, silica gel 60 F_{254}, CH_2Cl_2/AcOEt (98:2); UV (MeOH) λ_{max} (log ε) 279 (3.68); IR (KBr) ν_{max} 3650–3150, 3015, 2955, 1759, 1597, 1470, 1236 e 1186 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 6.26 (1H, d, J = 2.9 Hz, H-3), 6.20 (1H, d, J = 2.9 Hz, H-5), 3.73 (3H, s, OCH_3), 2.38 (2H, t, J = 7.7 Hz, H-1′), 2.29 (3H, s, $OCOCH_3$), 1.54–1.46 (2H, m, H-2′), 1.34–1.24 (4H, m, H-3′, H-4′), 0.86 (3H, t, J = 7.0 Hz, H-5′); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 170.2 (C, $OCOCH_3$), 151.3 (C, C-4), 150.0 (C, C-2), 136.1 (C, C-6), 131.2 (C, C-1), 107.6 (CH, C-5), 98.2 (CH, C-3), 55.8 (CH_3, OCH_3), 31.6 (CH_2, C-3′), 30.1 (CH_2, C-1′), 29.5 (CH_2, C-2′), 22.5 (CH_2, C-4′), 20.6 (CH_3, $OCOCH_3$), 14.0 (CH_3, C-5′); ESIMS m/z 252.1368 (calcd. for $C_{14}H_{20}O_4$ 252.1362) [M+], m/z 210 (100%) ($C_{12}H_{18}O_3$) [M-42].

Cell culture

K562 (acute myeloid leukemia, AML) and Jurkat (acute lymphoblastic leukemia ALL) cells were cultured in Roswell Park Memorial Institute medium (RPMI) (Gibco®, Massachusets, USA) supplemented with 10% inactivated fetal bovine serum (FBS). HeLa (cervix adenocarcinoma), HT-29 (colorectal adenocarcinoma), U251 (glioblastoma astrocytoma) and L929 cells (murine fibroblast) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco®, Massachusetts, USA) with 10% FBS. U251NS glioma stem-like cells were cultured in neurosphere medium based on DMEM/F12 supplemented with 20 ng/mL recombinant basic fibroblast growth factor (basic-FGF) (Invitrogen®, California, USA), 20 ng/ml recombinant epidermal growth factor (EGF) (Invitrogen®, California, USA) and 50 U/ml heparin (Sigma-Aldrich®, Missouri, USA). Cells were originally purchased from the American Type Culture Collection (ATCC) or the Rio de Janeiro Cell Bank (BCRJ).

Cell viability assays

Cells (5×10^4, 2.5×10^4 and 1.25×10^4 cells/well) were incubated for 24, 48 and 72 h with 1–50 μM of **MA**. The compound was previously dissolved in DMSO, which was maintained below 2% *v*/v. Viability was assessed by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium (MTT) method (Amresco®, Cleveland, USA) [22]. The optical density (OD) of untreated cells was considered as 100% of viable cells. The 50% inhibitory concentrations (IC_{50}) were obtained using GraphPad Prism 5 software. The selectivity index (SI) was calculated by dividing the IC_{50} obtained on normal cell line L929 and solid tumor cells by the IC_{50} obtained on AL cell lines. A high selectivity was considered as $SI > 10$, a moderate selectivity as $5 \leq IS \leq 10$ and a low selectivity as $IS < 5$ [23].

Peripheral blood (PB) and bone marrow (BM) collection

Peripheral blood (PB) samples were collected from five non-smoking healthy volunteers. In addition, two PB and three BM samples obtained from patients diagnosed with AL who attended the Hematology Service of the Polydoro Ernani de São Thiago University Hospital (Florianópolis, Brazil) were included in this study. Volunteers and patients signed an informed consent according to the ethics committee requirements (Medical Ethics Committee CEPSH n°746.486/2014) and, when applied, samples were collected before the first treatment. Mononuclear cells (MC) were isolated by Ficoll Hypaque (density.070 g/ml), viable cells were counted by the Trypan blue exclusion method and only samples with more than 90% cell viability were used. For the hemolysis test, red blood cells (RBC) were isolated b centrifugation from PB. Saline was used as negative control (0% lysis), distilled water as positive control (100% lysis) and **MA** at different concentrations (12, 24 and 36 μL). The hemolysis rate was calculated by the following equation: Hemolysis rate (%) = (Dt - Dnc) /(Dpc - Dnc) × 100, where: Dt, abs test sample; Dnc, abs negative control; Dpc, abs positive control [24].

Cell cycle analysis

K562 and Jurkat cells (1.0×10^6 cells/well) were treated with **MA** at the 24 h IC_{50} for 24 h and then fixed with 70% ethanol. Cells were incubated with PI/RNASE Kit Solution (Immunostep®, Salamanca, Spain), acquired by flow cytometry (BD FACSCantoTM II, Becton Dickinson Immunocytometry Systems®, New Jersey, USA) and analyzed by WinMID software version 2.8. The negative control was prepared with untreated cells and 20,000 gated events were acquired in the PE channel (510–595 nm).

Apoptosis assays

K562 and Jurkat cells (1.0×10^6 cells/well) were treated with **MA** for 12 h (24 h IC_{50}). Cells were resuspended in a solution of ethidium bromide (EB) (5 μg/mL) and acridine orange (AO) (10 g/mL) 1:1, observed in a fluorescence microscope (Olympus BX-FLA) using a 40x objective and representative fields were photographed with a digital camera (Olympus BX40, Japan). Phosphatidylserine exposure was evaluated using the Annexin V-FITC Apoptosis Detection Kit (Immunostep®, Salamanca, Spain) following the manufacturer's instructions. Analysis were performed by flow cytometry and analyzed by Infinicyt software version 1.7 (Cytognos®, Salamanca, Spain). For the DNA fragmentation assay, DNA extraction was performed using a commercial kit (QIAGEN®, Hilden, Germany) and DNA samples were separated by electrophoresis in 2% agarose gel stained with EB (1 μg/mL) and photographed under ultraviolet light (DOC-PRINT® Biosystems).

Mitochondrial membrane potential ($\Delta\psi_m$) and reactive oxygen species (ROS)

K562 and Jurkat cells (1.0×10^6 cells/well) were incubated with **MA** (24 h IC_{50}) and resuspended in MitoView 633 solution (Biotium®, San Francisco, USA) diluted 1:10.000 according to the manufacturer's instructions. ROS production was assessed by MitoSox Red Molecular Probes (Thermo Fischer®, Massachusetts, USA) and DRAQ7 (BioStatus®, Shepshed, UK). Analysis were performed in BD FACSCantoTM II flow cytometer and evaluated by Infinicyt software version 1.7. The negative control was prepared with untreated cells and 10,000 gated events were acquired.

Protein expression by FACS staining

K562 and Jurkat cells (1.0×10^6 cells/well) were treated with **MA** (24 h IC_{50}) for 12 h. Cells were permeabilized with BD Fix/Perm (BD® Biosciences, USA), except cells stained for Fas and PML expression, and incubated with anti-Bcl-2-FITC (Invitrogen®, USA), anti-Bax-PerCP (Santa Cruz Biotechnology®, USA), anti-AIF-FITC (Santa Cruz Biotechnology®, USA), anti-Ki-67-FITC (Santa Cruz Biotechnology®, USA), anti-survivin-PE (Dako®, USA) and anti-Fas-PE (Santa Cruz Biotechnology®, USA). For PML expression, cells were fixed in 4% paraformaldehyde and permeabilized with PBS 0.2% Triton X-100 before incubation with anti-PML (1:1000) (StressGen Biotechnologies®, British Columbia, Canada) and anti-mouse-Alexa 555 (Molecular Probes®, California, USA). Analysis was performed by flow cytometry and data was analyzed by Infinicyt software version 1.7. or FlowJo. The negative controls were prepared with untreated cells and the medium fluorescence intensity (MFI) was considered as 100% or 1.

Protein expression by Western Blot (WB)

K562 and Jurkat cells (1.0×10^6 cells/well) were treated with **MA** (24 h IC_{50}) for 24 h. Total cell lysates (TCL) were prepared with RIPA buffer containing protease inhibitors. In some cases cells were fractionated into cytosolic and mitochondrial fractions. The cytosolic fraction (CF) was obtained with cytosolic extraction buffer (70 mM KCl, 137 mM NaCl, 1.4 mM KH_2PO_4 pH 7.2, 4.3 mM Na_2HPO_4, 250 mM sucrose, 50 μg/mL digitonin, protease inhibitors). The pellet was further resuspended in a mitochondrial lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 2% Triton X-100, 0.3% NP-40, protease inhibitors) to isolate the mitochondrial fraction (MF). Protein concentration was determined by the BCA Protein Assay Kit (Thermo Fischer®, Massachusetts, USA) and 30 μg of protein per well were loaded on 12 or 15% SDS-PAGE gels. Proteins were transferred to blocked PDVF membranes (Bio-Rad®, California, USA) and incubated over night with primary anti-Caspase 3 (8G10) (Cell Signaling®, Danvers, USA), anti-PARP (H-250), anti-Tom40 (Santa Cruz Biotechnology®, Santa Cruz, USA), anti-cytochrome c (BD Biosciences®, New Jersey, USA), anti-Endo G (Novus Biological®, Littleton, USA), anti-GAPDH (Abcam®, Cambridge, UK). Membranes were then incubated with the secondary antibodies anti-rabbit IgG HRP and anti-mouse IgG HRP (Sigma-Aldrich®, Missouri, USA) and anti-goat IgG HRP (Santa Cruz Biotechnology Inc.®, Santa Cruz, USA) and immunoreactive proteins (bands) were revealed by the ECL developing system (Western Lightning Chemiluminescence Reagent Plus, Perkin-Elmer, Boston, MA).

Combined treatments

Miconidine acetate was evaluated in combination with cytarabine (Accord Healthcare®, Middlesex, UK) and vincristine (Libbs Pharmaceuticals®, São Paulo, Brazil), kindly donated by the Chemotherapy Sector of the University Hospital (Florianópolis, Brazil). K562 and Jurkat cells were treated with different concentrations of cytarabine or vincristine and then treated with miconidine acetate (IC_{50} and ½ IC_{50}) for 24 h.

Statistical analysis

Results are expressed as mean ± SD and all experiments were performed in triplicate and repeated at least three times independently. Statistical analysis was performed by paired *t* test or by analysis of variance using one way ANOVA, complemented with Bonferroni *post-hoc* test. A significance level of 5% was adopted in all analysis. Statistical analyzes were performed with GraphPad Prism 5 software.

Results

MA is potently cytotoxic against different tumor cell lines and against cancer stem cells, with lower IC_{50} values in AL cells

MA reduced the cell viability of AL cells (K562 and Jurkat) and solid tumor cells (HT-29, HeLA and U251) in a concentration- and time-dependent manner (Fig. 2a–g). The lower IC_{50} values were found in AL models, especially ALL Jurkat, in which values lower that 5 μM after 24 h and 1 μM after 48 and 72 h were obtained (Table 1). When treated solid tumor cells were compared to treated AL cells, a low selectivity was observed for K562 ($SI < 5$) and a moderate selectivity for Jurkat ($5 \leq IS \leq 10$) (Fig. 2h). Interestingly, **MA** was also able to reduce the viability of U251NS cancer stem-cells in a concentration- and time-dependent manner (Fig. 2e–g, Table 1).

MA is non-cytotoxic to non-tumor cells and non-hemolytic to normal RBCs

As shown in Fig. 2i, **MA** did not reduce the cell viability of fibroblasts, used as a model of non-tumor cells. The IC_{50} values were higher than 50 μM, meaning that they were non-significant when compared to the values observed in leukemic cells. In agreement with these results, the SI between treated L929 and treated K562 or Jurkat cells exceeded 10, which indicates a very high selectivity (Fig. 2h). When normal PBMCs were treated with **MA** (K562 IC_{50}), a significant reduction of 54.3% percent points (pp) was observed in cell viability (Fig. 2j). However, this effect was very similar to that observed by the widely used chemotherapeutic drug paclitaxel (58.5% pp). When **MA** was used at 4.7 μM (Jurkat IC_{50}), no significant reduction in cell viability was observed. In addition, **MA** did not cause significant hemolysis on normal RBCs even at concentrations 4 times higher than the IC_{50} calculated in K562 cells (less than 2% hemolysis when compared to the positive control) (Fig. 2k).

MA induces cell cycle arrest and apoptotic cell death in AL cells

As shown in Fig. 3a–b, **MA** blocked the G2/M phase in both K562 and Jurkat cells, as reflected by a significant increase in the percentage of cells in this phase (increase of 56% and 68% for K562 and Jurkat cells, respectively). **MA** also reduced the expression of cell proliferation marker KI67 ($MFI_{K562} = 0.74 \pm 0.05$; $MFI_{Jurkat} = 0.77 \pm 0.01$ (Fig. 3c) and increased PML expression (K562: 90%; Jurkat: 97.9%) in both cell lines (Fig. 3d). To evaluate the type of cell death induced by **MA** on AL cells, morphological changes in K562 and Jurkat cells were observed in a fluorescence microscope. After 12 h incubation, non-treated cells remained viable, with a uniform size and

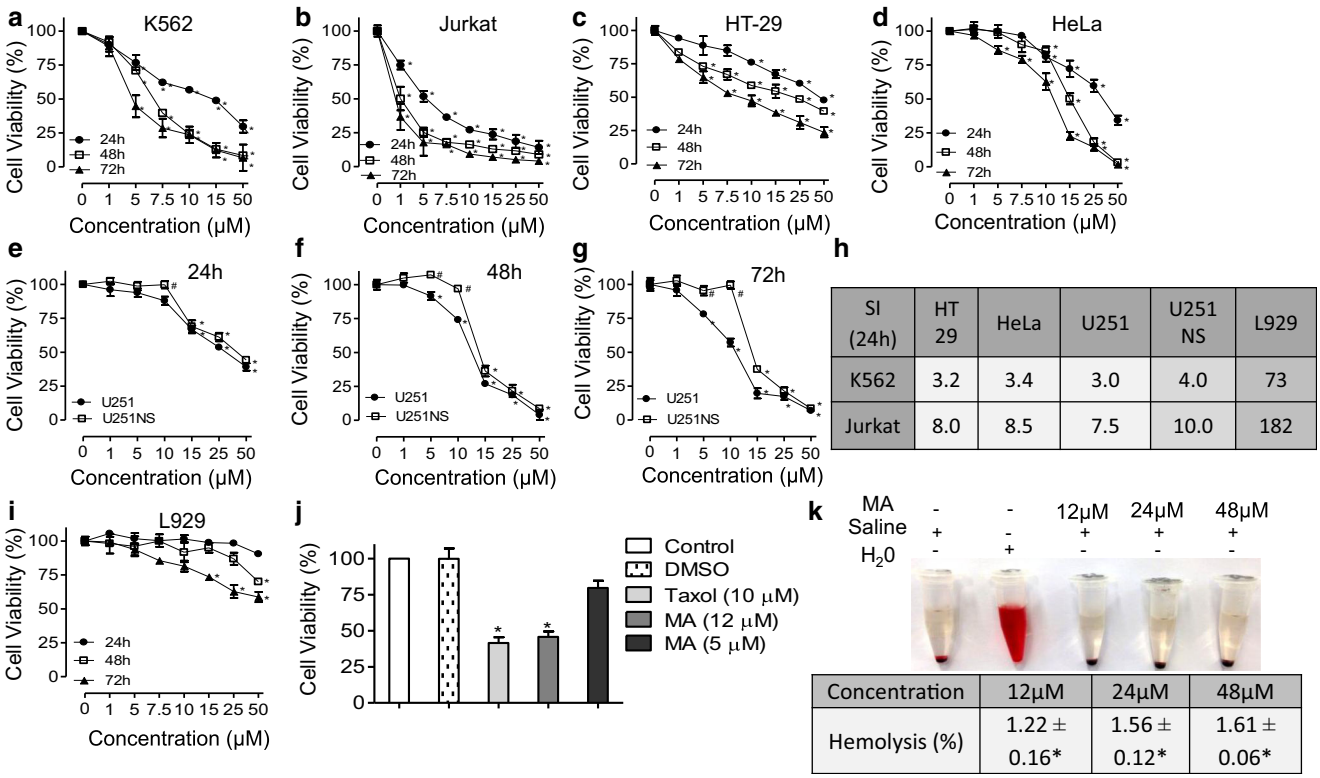

Fig. 2 Effect of MA on cancer cells and on normal cells. **a**–**d** Cytotoxic effect of MA on AL cells K562 (**a**) and Jurkat (**b**), and on solid tumor cells HT-29 (**c**) and HeLa (**d**). **e**–**g** Effect of MA on U251 glioma cells and on U251NS glioma stem cells. **h** Effect of MA on non-neoplastic cells L929. **i** Effect of MA on MC isolated from PB. DMSO was the solvent used and Taxol was included as a control. **j** Hemolysis test performed on RBC using destilled water as positive control (absorbance considered to be 100% hemolysis) and saline as negative control (absorbance considered as 0% hemolysis). * $p < 0.05$ when compared to the control group, *t*-test or one-way ANOVA fol

bright green color, while treated cells underwent cell shrinkage, chromatin condensation, pyknosis, bleb formation and loss of membrane integrity, which are characteristic of initial (I) and late (T) apoptosis (Fig. 3e). Apoptosis was confirmed by DNA fragmentation, as the "ladder pattern" was clearly observed in treated cells (Fig. 3f) and by caspase-3 and PARP cleavage by WB (Fig. 3g–h). Apoptotic cells were quantified by phosphatidylserine exposure and an increase of 1343% and 709% was observed in Annexin-V-positive K562 and Jurkat cells, respectively.

MA induces extended mitochondrial damage and activates both intrinsic and extrinsic apoptosis

Treatment with **MA** significantly reduced $\Delta\psi_m$ in K562 (41.6%) and Jurkat cells (57.0%) (Fig. 4a–c), which resulted in the release of apoptogenic factors, such as AIF, cytochrome c and endonuclease G (Fig. 4d–f). Furthermore, the compound induced mitochondrial ROS production in both AL cells (K562: 60.0 ± 3.4%; Jurkat: 63.5 ± 2.3%) (Fig. 3g–i). **MA** also increased the expression of the proapoptotic protein Bax ($MFI_{K562} = 1.9 \pm 0.1$; $MFI_{Jurkat} = 1.6 \pm 0.1$) (Fig. 4j), but no significant difference was observed in the expression of anti-apoptotic Bcl-2 (Fig. 4k). However, this modulation was sufficient to invert the Bax/Bcl-2 ratio in K562 (2.05) and Jurkat (1.78) cells, probably resulting in $\Delta\psi_m$ loss. Also, the compound decreased the expression of anti-apoptotic protein survivin (MFI_{K562} 0.85 ± 0.1; $MFI_{Jurkat} = 0.6 \pm 0.1$) (Fig. 4l) and increased FasR expression (MFI_{K562} 1.5 ± 0.1; MFI_{Jurkat} 1.3 ± 0.1), which indicates the concomitant involvement of extrinsic apoptosis (Fig. 4m). The modulation of these proteins altogether led to an increase in the expression of active caspase-3 in both AL cell lines (MFI_{K562} 1.53 ± 0.02; (MFI_{Jurkat} 1.66 ± 0.04) (Fig. 4n).

Table 1 IC_{50} calculated for MA on AL cells K562 and Jurkat, on solid tumor cells HT-29, HeLa, U251, on cancer stem cells U251NS and on L929 fibroblast

IC_{50} (μM)	K562	Jurkat	HT-29	HeLa	U251	U251NS	L929
24 h	11.7 ± 0.4	4.7 ± 0.3	37.4 ± 1.6	39.8 ± 1.5	35.2 ± 1.5	47.2 ± 1.5	854.0 ± 2.9
48 h	5.3 ± 0.3	0.9 ± 0.1	17.7 ± 1.8	18.6 ± 1.2	11.2 ± 1.5	18.2 ± 1.3	153.0 ± 2.1
72 h	3.6 ± 0.3	0.2 ± 0.1	9.4 ± 0.9	10.7 ± 1.3	9.3 ± 0.9	16.2 ± 1.2	51.6 ± 1.7

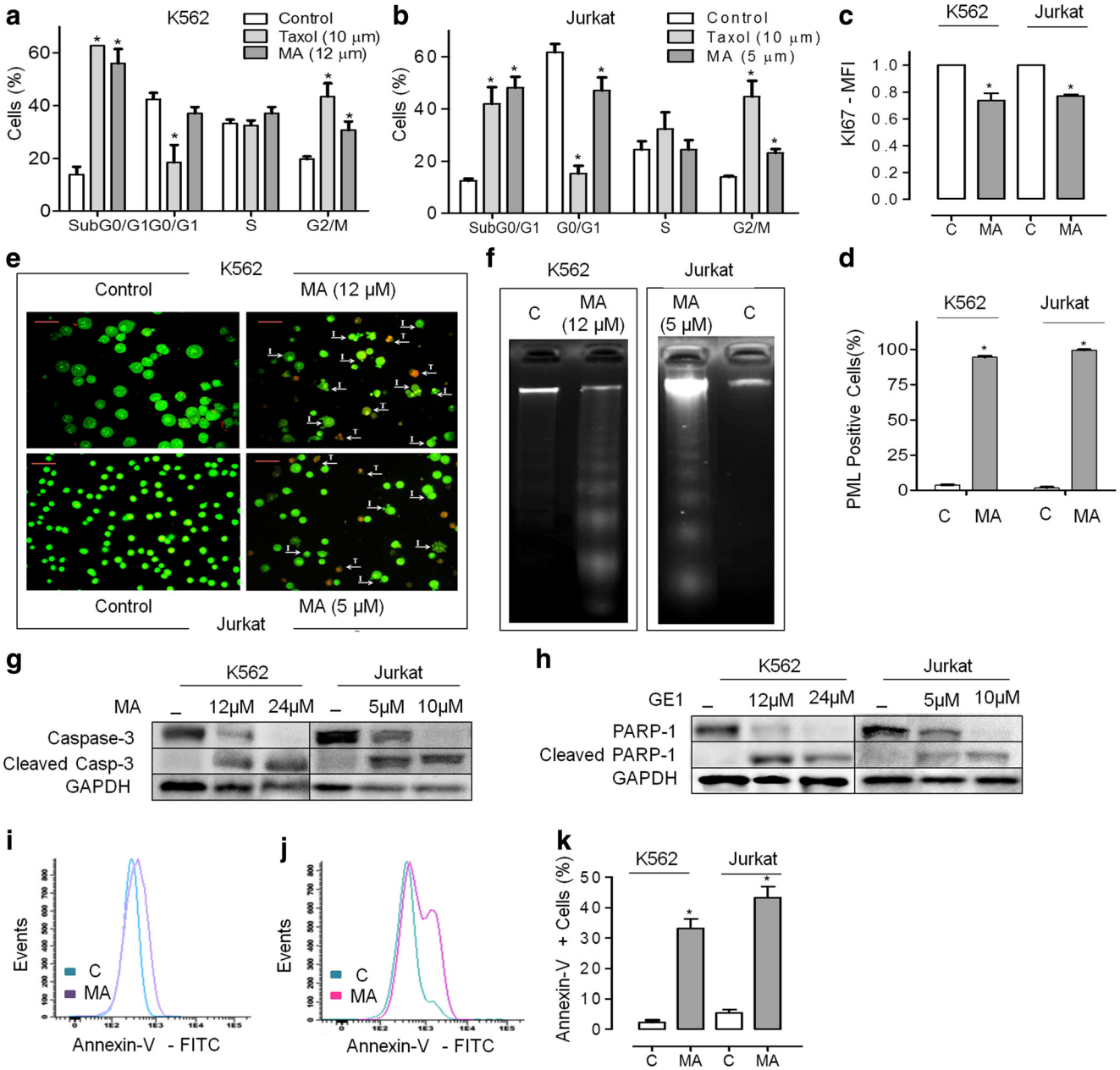

Fig. 3 Effect of miconidine acetate on cell cycle and apoptosis. **a**–**b** K562 (**a**) and Jurkat (**b**) cell cycle was evaluated after 24 h incubation with MA by PI staining and flow cytometry. Phases are represented by G0/G1, S, G2/M and sub-G0/G1. (C-D) Cells were labeled with anti-Ki67 (**c**) or anti-PML (**d**) and analyzed by flow cytometry (**e**) Cells were stained with EB/AO and observed in a fluorescence microscope. Apoptotic cells are indicated by the white arrows. **f** DNA fragmentation was evaluated in agarose gel. (G-H) Caspase-3 and PARP expression in K562 (**g**) and Jurkat cells (**h**) by WB. **i**–**k** Annexin-V positive K562 (**i**) and Jurkat cells (**j**) were quantified by flow cytometry. Each point and/or figure represents the mean ± SD of at least three independent experiments. * $p < 0.05$ when compared to the control group, *t*-test or one-way ANOVA followed by Bonferroni

MA is strongly cytotoxic against cells collected from patients with AL and enhanced the anti-leukemic effect of chemotherapeutics

After 24 h incubation, **MA** was able to reduce the cell viability of all PB and BM samples collected from AL patients in a concentration-dependent manner (Fig. 5a). The highest reduction in cell viability was observed in cells from Patient 4 (AML t(8;21)), while the lowest reduction was observed in cells from Patient 5 (APL) (Table 2). Combined treatment with chemotherapeutic cytarabine in K562 cells revealed that **MA** increased cell death by 42.4% (Cyt 1000 μM + IC_{50}), 31.1% (Cyt 1000 μM + 1/2 IC_{50}), 50.8% (Cyt 500 μM + IC_{50}) and 38.4% (Cyt 500 μM + 1/2 IC_{50}), when compared to cytarabine alone (1000 μM and 500 μM, respectively) (Fig. 5b). Similarly, the combined treatment of **MA** with vincristine in Jurkat cells

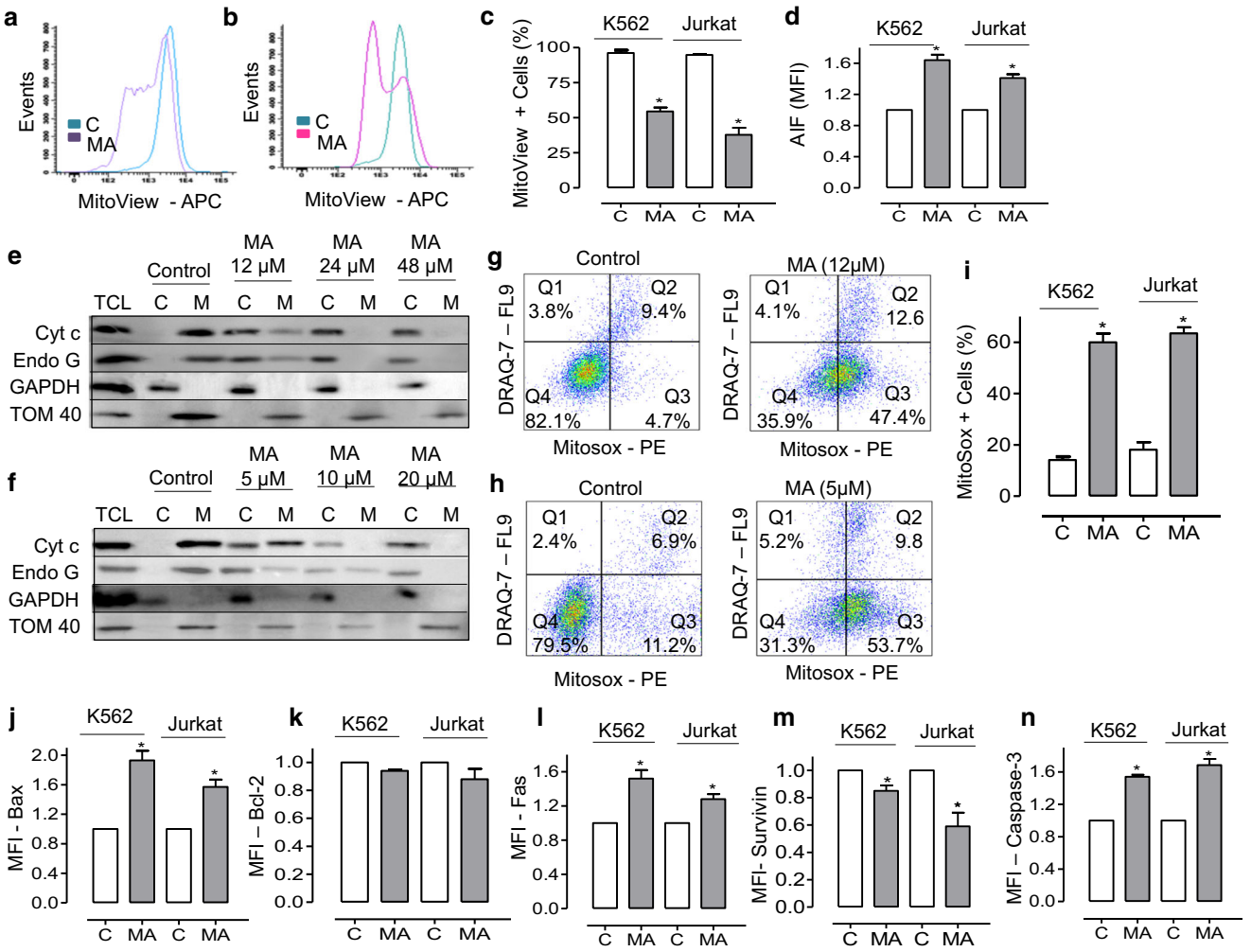

Fig. 4 Effect of miconidine acetate on the apoptotic machinery. **a**–**c** Treated K562 (**a**) and Jurkat (**b**) cells were incubated with Mitoview and the positive percentage was determined by flow cytometry. **d** Cells were labeled with anti-AIF-FITC and analyzed using MFI. **e**–**f** The cytosolic (**c**) and mitochondrial (**m**) fractions of K562 (**e**) and Jurkat cells (**f**) were obtained and samples were separated by WB. **g**–**i** ROS production was assessed in K562 (**g**) and Jurkat cells (**h**) by MitoSox Molecular Probes. **j**–**n** Treated K562 and Jurkat cells were labeled with anti-Bax-PerCP (**j**), anti-Bcl-2-FITC (**k**), anti-survivin-PE (**l**), anti-FasR-PE (**m**) and anti-caspase-3-FITC (**n**) and analyzed by flow cytometry. Results represent the mean ± SD of three independent experiments. * $p < 0.05$ when compared to the control group using *t*-test

increased the cell death in 26.2% (Vin 100 μM + IC_{50}), 20.3% (Vin 100 μM + 1/2 IC_{50}), 34.7% (Vin 50 μM + IC_{50}) and 37.7% (Vin 50 μM + 1/2 IC_{50}), when compared to vincristine alone (100 μM and 50 μM, respectively) (Fig. 5c).

Discussion

In this study, we extensively investigated the cytotoxic effects and death mechanisms of **MA** (Fig. 1), a promising new hydroquinone derivative. Since hematological tumors differ from solid tumors as they have different histological origins, pathogenic characteristics and clinical behavior [25], our results suggest that **MA** may be more effective against cells of hematological origin. This difference was expected given that chemotherapy in solid tumors is related to further challenges when compared to hematologic malignancies [26]. However, as previously demonstrated, the comparison between AL and solid tumor cells reveals that **MA** is moderately selective for ALL Jurkat and low selective for AML K562 cells (Fig. 2h), suggesting that even though a difference was observed, the compound was, in fact, cytotoxic against tumors of different origins. Additionally, in the last decades, studies have shown that ALs, similar to other malignancies, do not consist of a homogeneous cell population, but rather a heterogeneous mixture of stem cells, which maintain the disease, and slightly differentiated cells [27]. Cancer stem cells, located in both solid and hematological tumors, are highly tumorigenic, can survive and generally persist in tumors for a long period of time and are responsible for causing relapses, metastasis and the generation of new tumors [27, 28]. The fact that **MA** was also cytotoxic against U251NS glioma stem cells (Fig. 2e–g, Table 1) is extremely important given that

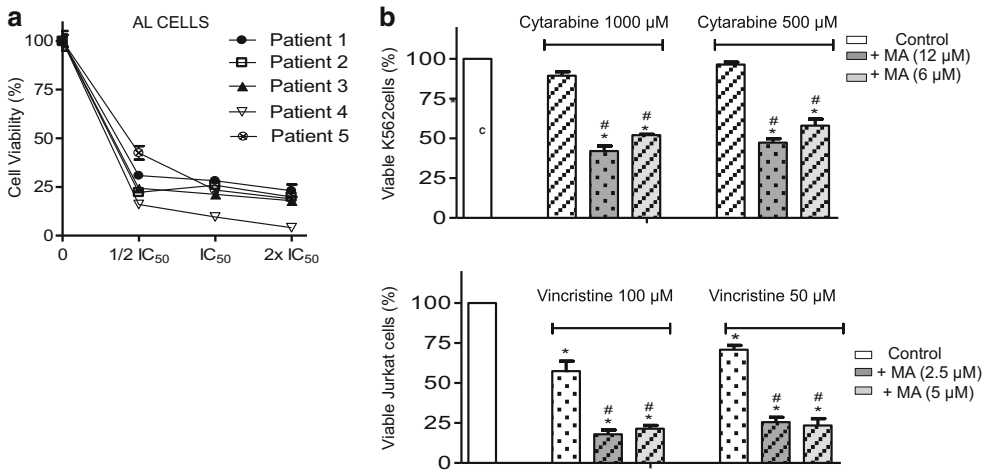

Fig. 5 Ex vivo and drug-combined experiments. **a** Cytotoxic effect of miconidine acetate on PB and MC cells collected from patients with AL before the first treatment. **b**–**c** K562 (**b**) and Jurkat cells (**c**) were treated with miconidine acetate (IC_{50} and 1/2 IC_{50}) and with different concentrations of cytarabine and vincristine. Untreated cells were considered as control groups. Results represent the mean ± SD of three independent experiments. * $p < 0.05$ when compared to the control groups, # $p < 0.05$ when compared to cytarabine or vincrsitine alone, *t* test or one-way ANOVA followed by Bonferroni

these cancer stem-cells are usually more resistant to treatment. Furthermore, in clinics, complete remission and long-term cure can only be achieved when these cells are eliminated, since they are involved in minimal residual disease [29, 30].

The search for new chemotherapeutic compounds is based not only on their ability to induce tumor cells to die, but also on their selectivity, in order to to minimally affect normal cells [31, 32]. According to the literature, the SI calculation is essential in the investigation of new drugs and should always be performed before phase 1 clinical studies [33]. The SI comparing L929 cells to AL cell lines was higher than 70, which indicates that **MA** is at least 70 times more cytotoxic to AL than to normal cells. Finally **MA** did not cause significant hemolysis, even at concentrations three times higher than the higher IC_{50} observed in AL cells (Fig. 2j). The evaluation of erythrocyte membrane stability is a good indicator of damage caused by a new compound; therefore, it is a useful screening test in cytotoxicity studies [34, 35]. This result might also indicate that **MA** could be administered intravenously.

Previous studies have shown that the cytotoxicity of antineoplastic compounds is related to the discontinuation of cell cycle progression and/or to the induction of programmed cell death [36, 37]. We have demonstrated that **MA** significantly increased the proportion of AL cells in the sub-G0/G1 phase, as previously reported in the KG-1a cell line [38]. In addition, **MA** blocked the cell cycle of both K562 and Jurkat cells in the G2/M phase, similar to paclitaxel [39]. The G2/M blockage is usually related to microtubule interference due to the compound's ability to bind to tubulin [40]. The molecular mechanisms of cell cycle regulation in cancer are disrupted by mutations in major genes involved in cell cycle checkpoints. The PML protein is expressed in most normal human tissues and plays an important role in gene regulation and in cellular processes such as DNA repair, cell cycle control and cell death [41]. Recently, it was reported that PML up-regulation is critical for dexamethasone-mediated leukemic cell death [42, 43]. Moreover there is a direct correlation between the susceptibility to this glucocorticoid and PML expression in leukemia

Table 2 Patient's information and results

Patient	Age (years)	Sample	Diagnosis	Blast cells (%)	Cell viability % (1/2 IC_{50})	Cell viability % (IC_{50})	Cell viability % (2x IC_{50})
1	81	PB	Early T cell precursor ALL	76%	30.8 ± 1.9	28.2 ± 0.7	23.1 ± 2.3
2	56	PB	AML without maturation	83%	22.2 ± 0.5	25.9 ± 0.8	10.9 ± 0.2
3	67	BM	AML secondary to MDS	38%	24.4 ± 0.2	21.2 ± 0.7	17.9 ± 0.7
4	33	BM	AML t(8;21)	27%	15.9 ± 1.8	9.5 ± 0.8	4.1 ± 0.2
5	31	BM	PML t(15;17) Bcr3+ FLT3 DIT+	96%	42.5 ± 3.5	23.4 ± 1.8	18.8 ± 0.4

Clinical information regarding the samples of five AL patients included in this study, as well as the cell viability percentage calculated after treatment of isolated MC with miconidine acetate at three increasing concentrations

patients [44]. In this paper, **MA** increased PML expression, which suggests that it acts similarly to dexamethasone in AL cells. **MA** also reduced the expression of the cell proliferation marker KI67 on both K562 and Jurkat cells, which indicates that the compound also has some cytostatic effects.

Cell cycle blockage aims to repair DNA in stress situations; if the repair does not occur properly, several pathways lead to the activation of programmed cell death. We first observed apoptotic characteristics in K562 and Jurkat cells treated with **MA**, and apoptosis was further confirmed by DNA fragmentation, phosphatidylserine exposure and caspase-3 and PARP cleavage (Fig. 3g–h). Additionally, **MA** induces pronounced mitochondrial damage, as initially reflected b $\Delta\psi_m$ loss in more than 50%. According to the literature, $\Delta\psi_m$ perturbation leads to increased membrane permeability and, consequently, to the release of apoptogenic proteins such as AIF, cytochrome c and endonuclease G to the cytosol (Fig. 4d–f) [45–47]. ROS production after **MA** treatment (Fig. 3h–j) most likely backfired to potentiate the release of apoptogenic factors, as it was previously demonstrated that ROS is necessary for their proper release [48]. Mitochondrial permeabilization and, consequently, the release of mitochondrial proteins are regulated by the Bcl-2 family, which is composed of pro-apoptotic and anti-apoptotic members. The balance between these proteins keeps cells alive and, therefore, the inverse proportion of pro-apoptotic and antiapoptotic members is responsible for the induction of apoptotic cell death [49]. Apoptosis may also be related to the decreased expression of caspase-inhibiting proteins, such as survivin. Lower levels of this protein are related to apoptosis and, in clinics, to a better response to treatment [50]. **MA** significantly increased the pro-apoptotic protein Bax, resulting in Bcl-2/Bax inversion, and reduced the anti-apoptotic protein survivin (Fig. 4j–l). In addition, **MA** increased FasR expression in both K562 and Jurkat cells, which indicates that it also triggers extrinsic apoptosis. The increased expression of active-caspase-3, as well as caspase-3 and PARP cleavage, shows that apoptosis is not only being triggered but also executed.

In view of the promising results observed for **MA** in immortalized leukemic cell lines, we decided to evaluate its effect on blast cells obtained from PB or BM of five patients suffering from different AL subtypes (Fig. 5a, Table 2). In-vitro, **MA** was cytotoxic against both ALL (Jurkat) and AML (K562) cells, and similar results were observed ex vivo, as **MA** reduced the cell viability of patients' MC in a concentration-dependent manner. While **MA** was almost three times more cytotoxic against ALL Jurkat than AML K562 cells, the compound was able to reduce the viability of ALL and AML patients' cells in a very similar manner. The lower reduction in cell viability was observed in a patient with acute promyelocytic leukemia (APL) (Patient 5). According to the literature, APL is a more differentiated AML [51], which might indicate that **MA** is more active against more immature hematological diseases. Even though the number of samples was small, these results are very promising and further studies should be carried out in order to establish a real profile of **MA** on patients' AL cells.

Finally, the concept of therapeutic association is based on the synergistic or additive potential of two or more drugs aiming to improve their therapeutic efficacy, reduce side effects and also prevent the development of resistance to individual components. The combination of **MA** with the currently used chemotherapeutic drug cytarabine at sub-concentrations reduced K562 cell viability by almost 50%. This reduction was higher than those observed in cells treated with higher doses of cytarabine (10.6% reduction) or **MA** (25% reduction). Similarly, in Jurkat cells, vincristine cytotoxic effect was induced at a lower concentration by the addition of **MA** at the ½ IC_{50}. This combination resulted in an almost 80% reduction in cell viability, while cells treated with higher doses of vincristine or **MA** alone had a reduction of 42% and 25%, respectively. These results may indicate that **MA** sensitizes K562 and Jurkat cells to cytotoxicity induced by cytarabine and vincristine at lower doses, suggesting a complementary effect. This combination might result in the reduction of side effects caused by the two chemotherapeutics, as well as in resistance prevention in patients treated with both drugs.

Altogether, our results show that **MA**, a natural compound isolated from *E. hiemalis*, is strongly cytotoxic not only against AML and ALL, but also against solid tumor cells and cancer stem cells, yet with no significant toxicity to normal cells. The mechanisms of cell death induced by **MA** involves G2/M blockage with PML expression, decreased cell proliferation, ROS generation and intrinsic and extrinsic apoptosis, leading to mitochondrial damage, the release of pro-apoptotic mitochondrial proteins, caspase 3 and PARP activation and DNA fragmentation. Taken together, our results indicate that **MA** might be a promising novel compound for the treatment of a wide range of cancers, especially AL.

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Author contributions Mariana Maioral: Cell viability, FACS and WB experiments; manuscript writing.

Natália Stefanes: Synergic experiments with chemotherapeutics; manuscript writing.

Álisson Bigolin: Cell viability experiments; manuscript writing.

Gabriele Zatelli: Compound extraction, isolation and identification; manuscript writing.

Ana Philippus: Compound extraction, isolation and identification; manuscript writing.

Miriam de Barcellos Falkenberg: Manuscript review; experimental supervision.

Maria Cláudia Santos-Silva: Manuscript review; experimental supervision.

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Compliance with ethical standards

Conflict of interest Author A (Mariana Franzoni Maioral) declares that she has no conflict of interest. Author B (Natália Marceli Stefanes) declares that she has no conflict of interest. Author C (Álisson Bigolin) declares that he has no conflict of interest. Author D (Gabriele Andressa Zatelli) declares that she has no conflict of interest. Author E (Ana Cláudia Philippus) declares that she has no conflict of interest. Author F (Miriam de Barcellos Falkenberg) declares that she has no conflict of interest. Author G (Maria Cláudia Santos-Silva) declares that she has no conflict of interest.

Ethical approval All procedures performed in this study involving human participants have been conducted with the ethical approval of relevant bodies and that such approvals are acknowledged within the manuscript (Medical Ethics Committee CEPSH n°746.486/2014).

Informed consent Informed consent was obtained from all individual participants included in the study.

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