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Sensitization of K562 Leukemia Cells to Doxorubicin by the *Viscum album* Extract

Tatjana Srdic-Rajic[1†] Nevena Tisma-Miletic,[1†] Milena Cavic,[1] Ksenija Kanjer,[1] Katarina Savikin[2] Danijel Galun[3,4] Aleksandra Konic-Ristic[5] and Tamara Zoranovic[1,6]*

[1]Department of Experimental Pharmacology, Institute for Oncology and Radiology of Serbia, Belgrade, Serbia
[2]Institute for Medicinal Plant Research 'Dr Josif Pančić', Belgrade, Serbia
[3]University Clinic for Digestive Surgery, Clinical center Serbia, Belgrade, Serbia
[4]Belgrade University Medical School, Belgrade, Serbia
[5]Institute for Medical Research, Center of Research Excellence in Nutrition and Metabolism, Belgrade University, Belgrade, Serbia
[6]Max Plank Institute for Infection Biology, Berlin, Germany

Toxicity of conventional chemotherapeutics highlights the requirement for complementary or alternative medicines that would reduce side effects and improve their anticancer effectiveness. European mistletoe (*Viscum album*) has long been used as a complementary and alternative medicine supporting cancer therapy. The aim of this study was to investigate synergistic antitumor action of *V. album* extract and doxorubicin during co-treatment of chemoresistant chronic myelogenic leukemia K562 cells. Combined treatment of leukemia cells led to inhibitory synergism at sub-apoptotic doxorubicin concentrations and multifold reduction of cytotoxic effects in healthy control cells. Prolonged co-treatment was associated with reduced G2/M accumulation and increased expression of early and late apoptotic markers. Our data indicate that *V. album* extract increases antileukemic effectiveness of doxorubicin against resistant K562 cells by preventing G2/M arrest and inducing apoptosis. **Copyright © 2015 John Wiley & Sons, Ltd.**

Keywords: Viscum album; doxorubicin; K562 leukemia cells; complementary medicines; therapy; apoptosis.

Abbreviations: CAMs, complementary or alternative medicines; CML, chronic myeloid leukemia; Dox, doxorubicin; VAE, *Viscum album* extract; MDR, multidrug resistant; BSA, bovine serum albumin; Δψm, mitochondrial transmembrane potential.

INTRODUCTION

European mistletoe (*Viscum album*) has been used for centuries in traditional medicine to treat seizures, headaches, and other conditions. Today, mistletoe extract [*V. album extract* (VAE)] therapy is among the most commonly used and thoroughly studied complementary treatments in Europe (Beuth and Schierholz, 2007; Fasching *et al*., 2007; Molassiotis *et al*., 2005, 2006a, 2006b). Many kinds of metabolites have been isolated from the European mistletoe, and some are synthesized by the host and incorporated into *V. album* (Cordero *et al*., 1993; Giudici *et al*., 2004). Viscotoxins and amphipathic and basic polypeptides (Schaller *et al*., 1998); tyramine, phenylethylamine, choline, and acetylcholine (Hegnauer, 1966); aminoalkaloids (Amer *et al*., 2012); phenolic acids and flavonoids (Deliorman *et al*., 2002; Fukunaga *et al*., 1987; Luczkiewicz *et al*., 2001; Vicas *et al*., 2011); phenylpropanoids (Panossian *et al*., 1998; Wagner *et al*., 1986); terpenoids (Fukunaga *et al*., 1987); long-chain fatty acids and hydrocarbons as well as trace amounts of volatile components including *trans*-a-bergamotene, *trans*-b-farnesene, loliolide, and vomifoliol (Cebović *et al*., 2008); and oligosaccharides and polysaccharides (Arda *et al*., 2003; Jordan and Wagner, 1986) belong to active metabolites isolated from the mistletoe.

The main group of *V. album* metabolites with described anticancer activity are lectins. Mistletoe lectins (ML-I, ML-II, and ML-III) are classified as type II ribosome-inactivating proteins (Wacker *et al*., 2004). The European mistletoe obtained from deciduous trees contains mostly ML-I and, when grown on some host such as pine and fir trees, ML-III.

Viscum album extract preparations have been tested in clinical trials as supporting medicines for cancer surgery, chemotherapy, or radiotherapy providing support for their therapeutically beneficial effects (Augustin *et al*., 2005; Bussing *et al*., 2005; Kienle *et al*., 2003; Kienle and Kiene, 2010; Klopp *et al*., 2005; Melzer *et al*., 2009; Ostermann *et al*., 2009; Schink *et al*., 2007; Troger *et al*., 2009, 2013, 2014a, 2014b). Functional studies suggest that VAEs, when administered to tumor-bearing animals, display growth-inhibiting and tumor-reducing effects (Büssing, 2000; Duong Van Huyen *et al*., 2002; Kienle and Kiene, 2003; Park *et al*., 2001). Furthermore, total VAEs have been reported to have additive inhibitory interactions with anticancer drugs by enhancing their cytostatic and cytotoxic effects (Bantel *et al*., 1999; Siegle *et al*., 2001). These effects are, at least in part, attributed to strong cell death-inducing properties of MLs (Bussing and Schietzel, 1999; Eggenschwiler *et al*., 2007; Elsasser-Beile *et al*., 1998). This strong proapoptotic activity of VAEs has been suggested to play a role in elimination of multidrug-resistant (MDR) colon cancer cells (e.g., MDR+ colon cancer cells) (Valentiner *et al*., 2002). Beside extensive experimental analyses of their biological properties, many questions related to the precise mode of action of VAE still remain unanswered.

* Correspondence to: Tamara Zoranovic, Department of Molecular Biology, Max Planck Institute for Infection Biology, Campus Charité Mitte, Charitéplatz 1, D-10117 Berlin, Germany.
E-mail: zoranovic@mpiib-berlin.mpg.de
†These authors contributed equally to this work.

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Despite great advancements in the area of individualized medicine, chemotherapeutics often remain a therapy of choice especially for advanced malignancies (Jackson and Chester, 2015). Because of their unselective mode of action, numerous side effects arise during therapy, significantly contributing to cancer patient fatalities (Dicato, 2012). Therefore, one of the major challenges in cancer chemotherapy today is the need to reduce doses of therapeutics while maintaining their optimal anticancer activity. Effective doses of chemotherapeutics are defined by relative resistance/sensitivity of cancer cells often determined by a set of specific mutations (Marquette and Nabell, 2012; Martin *et al.*, 2014). Chronic myeloid leukemia (CML) is characterized by a reciprocal chromosomal translocation between chromosomes 9 and 22 (t(9; 22) (q34; q11)), giving rise to *Bcr-Abl* oncogene (Johansson *et al.*, 2002). Constitutively activated *Bcr-Abl* tyrosine kinase plays a critical role in the pathogenesis of CML and therapeutic resistance (Daley and Baltimore, 1988; Lugo *et al.*, 1990; Sattler and Salgia, 1997). In order to reduce side effects and improve effectiveness of standard therapeutics, many complementary or alternative medicines (CAMs) have been tested so far with differing effectiveness (Beuth and Schierholz, 2007; Marvibaigi *et al.*, 2014; Son *et al.*, 2010).

The aim of this study was to investigate possible additive antitumor activity of VAE with doxorubicin (Dox) and gain insight into molecular processes underlining these effects.

MATERIALS AND METHODS

Drug. Doxorubicin hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) was diluted in sterile water.

Preparation of mistletoe extract. Leaves of European mistletoe (*V. album* L.) were harvested from plants growing on apple trees (*Malus domestica* B.) in the western regions of Serbia. Voucher specimen no. 3655 HFF is housed in herbarium collection of the University of Belgrade, Faculty of Pharmacy, Department for Botany, Serbia.

Water extracts were obtained from milled fresh leaves with a solvent : solid phase ratio of 20:1 (v/w), by ultrasound-assisted extraction (UAE; 20 min; 360 W). Obtained mixture was centrifuged (2000 rpm/10 min) and subsequently filtered through 0.45-µm pore-sized membrane (Whatman, UK). Obtained extracts were analyzed for total protein and total phenolic content, in triplicates. Total protein content of the extract, determined by the Bradford method (Kruger, 1994), was 404 ± 12 µg/mL with bovine serum albumin (BSA) used for standard curve calibration (100–1000 µg/mL). Total phenolic content was 313 ± 9 µg gallic acid equivalents per milliliter determined by the Folin-Ciocalteu method (Dewanto *et al.*, 2002; Wolfe *et al.*, 2003) with gallic acid used for standard curve calibration (100–1000 µg/mL).

Cell line. The culture conditions for human chronic myelogenic leukemia cell line, K562 (American Type Culture Collection, Rockville, MD, USA), were as described earlier (Eshkourfu *et al.*, 2011).

Cytotoxicity assay. The cytotoxic activity of VAE and Dox on K562 cells was assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2,5-dyphenyl tetrazolium bromide (MTT) assay (Mosmann, 1983; Ohno and Abe, 1991). After treatment in 96-well plates, the MTT solution (Sigma-Aldrich, St. Louis, MO, USA) (20 µL/well) was added to each well. Samples were incubated for a further 4 h, followed by the addition of 100 µL of 10% SDS. Absorbance at 570 nm was measured the next day.

Cell survival (S %) was calculated as an absorbance (A570 nm) ratio between treated and control cells multiplied by 100. Half-maximal inhibitory concentration (IC_{50}) was defined as the concentration of the agent that inhibited cell survival by 50% compared with the vehicle control.

Flow cytometric analysis of cell cycle phase distribution. Briefly, 2×10^5 cells/Petri dish (dimensions 60 × 15 mm, NUNC) were treated with investigated compounds as indicated. After collection, cells were fixed with ethanol and stained with propidium iodide (PI) (Sigma-Aldrich, St. Louis, MO, USA). Cell cycle phase distribution was analyzed by FACSCalibur Becton Dickinson flow cytometer using the CELL QUEST computer software (Becton Dickinson, Heidelberg, Germany).

Apoptotic assay. Apoptotic rates were assessed with flow cytometry using the Annexin V–fluorescein isothiocyanate/PI kit (BD Pharmingen, San Diego, CA, USA). Samples were prepared according to manufacturer's instructions. Flow cytometry analysis was performed using a FACSCalibur cytometer using the CELL QUEST computer software (Becton Dickinson, Heidelberg, Germany).

Quantification of mitochondrial transmembrane potential. Mitochondrial transmembrane potential ($\Delta\psi m$) was measured using a cationic fluorochrome rhodamine 123 (Rh123) (Sigma-Aldrich, St. Louis, MO, USA) as described by Yan *et al.* (2007). Briefly, 1×10^6 cells resuspended in 200 µL of phosphate-buffered saline (PBS) were stained with Rh123 (2.5 µg/mL) for 30 min at 37 °C. After washing, samples were analyzed by flow cytometry using CELL QUEST software (Becton Dickinson, Heidelberg, Germany).

Flow cytometric analysis of cyclins B1 and D1 expression. Cells stained for fluorescence-activated cell sorting (FACS) analysis were treated as described earlier. For intracellular cyclin staining, the following antibodies were used: fluorescein isothiocyanate (FITC)-conjugated mouse anti-human cyclin B1(BD Pharmingen, San Diego, CA, USA), FITC-conjugated mouse anti-human cyclin D1 antibodies (BD Pharmingen, San Diego, CA, USA), and IgG2a isotype controls (BD Pharmingen, San Diego, CA, USA). Briefly, cells were incubated with

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antibodies overnight at 4 °C and washed twice with PBS containing 1% BSA. Cell pellets were resuspended in PBS/PI/DNase-free RNase A and incubated in the dark at room temperature for 30 min before acquisition. Samples were analyzed on a FACSCalibur cytometer using the CELL QUEST software (Becton Dickinson, Heidelberg, Germany).

Flow cytometric analysis of apoptotic markers. Cells stained for FACS analysis were treated as described earlier. For detection of apoptotic cells, the following antibodies was used: mouse anti-Bax (BD Pharmingen, San Diego, CA, USA, 1:1000), FITC-conjugated monoclonal active caspase-3 antibody (BD Pharmingen, San Diego, CA, USA, 1:1000). Briefly, cells were incubated with antibodies for 30 min at RT and washed twice with PBS containing 1% BSA. Cell pellets were resuspended in PBS and analyzed on a FACSCalibur cytometer using the CELL QUEST software (Becton Dickinson, Heidelberg, Germany).

Analysis of gene expression by real-time polymerase chain reaction. Total RNA was isolated using a TRI REAGENT® BD kit (Sigma-Aldrich, St. Louis, MO, USA). cDNA synthesis from total RNA using random primers and MultiScribe™ Reverse Transcriptase from a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA). All target transcripts (Bax and Casp3) were detected using TaqMan® Gene Expression Assays (Applied Biosystems, Carlsbad, CA, USA). All reactions were performed in duplicate, and the data represent mean ± SEM of three independent experiments. Results were analyzed using the classical delta-delta-Ct method. GraphPad Prism 5.04 (GraphPad Software, San Diego, CA, USA) was used to compare means by two-way analysis of variance and Bonferroni method to adjust the p-value for multiple comparisons. Differences were considered significant if $p < 0.05$. p-Values for each analysis are indicated in figure legends.

Clonogenic assay for proliferation ability. Briefly, 0.5×10^4 treated K562 cells per milliliter were mixed with 1 mL of Human methylcellulose complete medium (R&D Systems Minneapolis, MN, USA). Cells were then plated in a 35-mm plastic dish (Falcon). Blast-derived colonies (>50 cells) were counted on day 7. All assays were performed in duplicate.

Data analysis. Cytotoxicity of investigated substances was evaluated using Student's t-test, with $p < 0.05$ considered as statistically significant. Data obtained from the mistletoe–drug interaction were analyzed by CALCUSYN, a statistical program used for the assessment of drug interaction based on the method of Chou and Talalay (1984). The type of interaction, synergism, antagonism, or additive effect, was assessed based on the obtained combination index (CI).

RESULTS

Viscum album extract treatment affects chronic myeloid leukemia cell viability

For many CML patients, first-line therapy failure leads to the blast phase of the disease where employment of chemotherapeutics such as Dox is indicated. As *Bcr-Abl*-positive K562 leukemia cells are relatively resistant to Dox (Blagosklonny *et al*., 2001; Fang *et al*., 2000), we have investigated the potential benefit of using VAE as a supporting drug during Dox treatment. A series of treatment and co-treatment experiments were performed on K562 cells using a defined range of concentrations for both Dox and VAE. First, we tested the toxicity of five different VAE concentrations (16.0, 8.0, 4.0, 2.0, and 1.0 µg/mL of total proteins) on healthy peripheral blood mononuclear cells (PBMCs) using the MTT assay (Mosmann, 1983; Ohno and Abe, 1991). Approximately 80% of cells survived the 72-h treatment with VAE concentrations of 4, 2, and 1 µg/mL and IC_{50} value of 13.36 ± 1.6 µg/mL (data not shown). In addition, VAE IC_{50} value for K562 cells amounted to 0.5 ± 0.08 µg/mL, revealing approximately 30 times lower VAE toxicity on healthy PBMCs compared with K562 leukemia cells.

Half-maximal inhibitory concentration value for Dox treatment of K562 cells amounted to 0.7 µg/mL. Based on this result, we have selected three more Dox concentrations falling within a therapeutically relevant range (2 and 1 µM) or significantly below it (0.5 and 0.25 µM). Next we have determined K562 cell viability after 72-h treatment with VAE, Dox, or a combination of both using selected concentrations of both agents. We have observed that VAE treatment of K562 cells at all chosen concentrations exhibits antitumor activity in a dose-dependent manner (Table 1). Moreover, VAE treatment-mediated toxicity of K562 cells was comparable with those induced by Dox alone (Table 1). Consistent with previous observations, K562 leukemia cells exhibited pronounced resistance to Dox treatment (Blagosklonny *et al*., 2001; Fang *et al*., 2000) reflected by only a marginal increase in cell death at high/therapeutic Dox concentrations (Table 1). Surprisingly, despite dose-dependent toxicity observed during single treatments with VAE or Dox, co-treatment of K562 cells with a high concentration of VAE and Dox did not reveal synergistic, but rather mild antagonistic, action (Table 1, columns I and II; Fig. 1A and B). Much to our surprise, co-treatment within low/sub-therapeutic concentrations of both Dox and VAE significantly affected K562 cell survival, revealing a strong synergistic action: CI = 0.488, synergism; CI = 0.574, synergism (Table 1, columns III and IV; Fig. 1A and B). This synergistic antitumor activity at non-toxic concentrations of Dox and VAE was comparable with that induced by high Dox concentrations (Table 1, columns I and II). The same results were obtained by *in vitro* cell survival assay, or clonogenic assay. The growth inhibitory effect of VAE was already detected at 0.8 µg/mL (Fig. 1C), while VAE at 1.6 µg/mL was as potent as 0.5 µM Dox to reduce the number of colonies capable of growing in methylcellulose (Fig. 1C). Moreover, colony formation of K562 cells was more effectively inhibited by the combination of VAE and Dox (Fig. 1C). Together, our results reveal that co-treatment of K562 leukemia cells with VAE and

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Table 1. Dose-dependent cytotoxicity of doxorubicin (Dox), mistletoe extract (VAE), and VAE/Dox combined treatment of K562 leukemia cells (expressed as percentage of killed cells)

Substance	Drug concentration I	II	III	IV
VAE	71.52 ± 3.5	69.05 ± 2.45	57.27 ± 2.3	49.34 ± 2.6
Dox	63.60 ± 2.5	53.62 ± 3.7	42.55 ± 2.6	31.50 ± 2.9
VAE + Dox	74.31 ± 2.3	72.04 ± 2.8	69.78 ± 1.1	64.33 ± 1.2
CI	1.347	0.838	0.517	0.414
Interaction	− −	+	+ + +	+ + +

VAE (μg/mL): I = 4.0; II = 2.0; III = 1.0; IV = 0.5. Dox (μM): I = 2.0; II = 1.0; III = 0.5; IV = 0.25. The results are presented as mean (from three independent experiments) ± standard error of the mean. Interaction: + + +, synergism; +, slight synergism; − −, moderate antagonism.

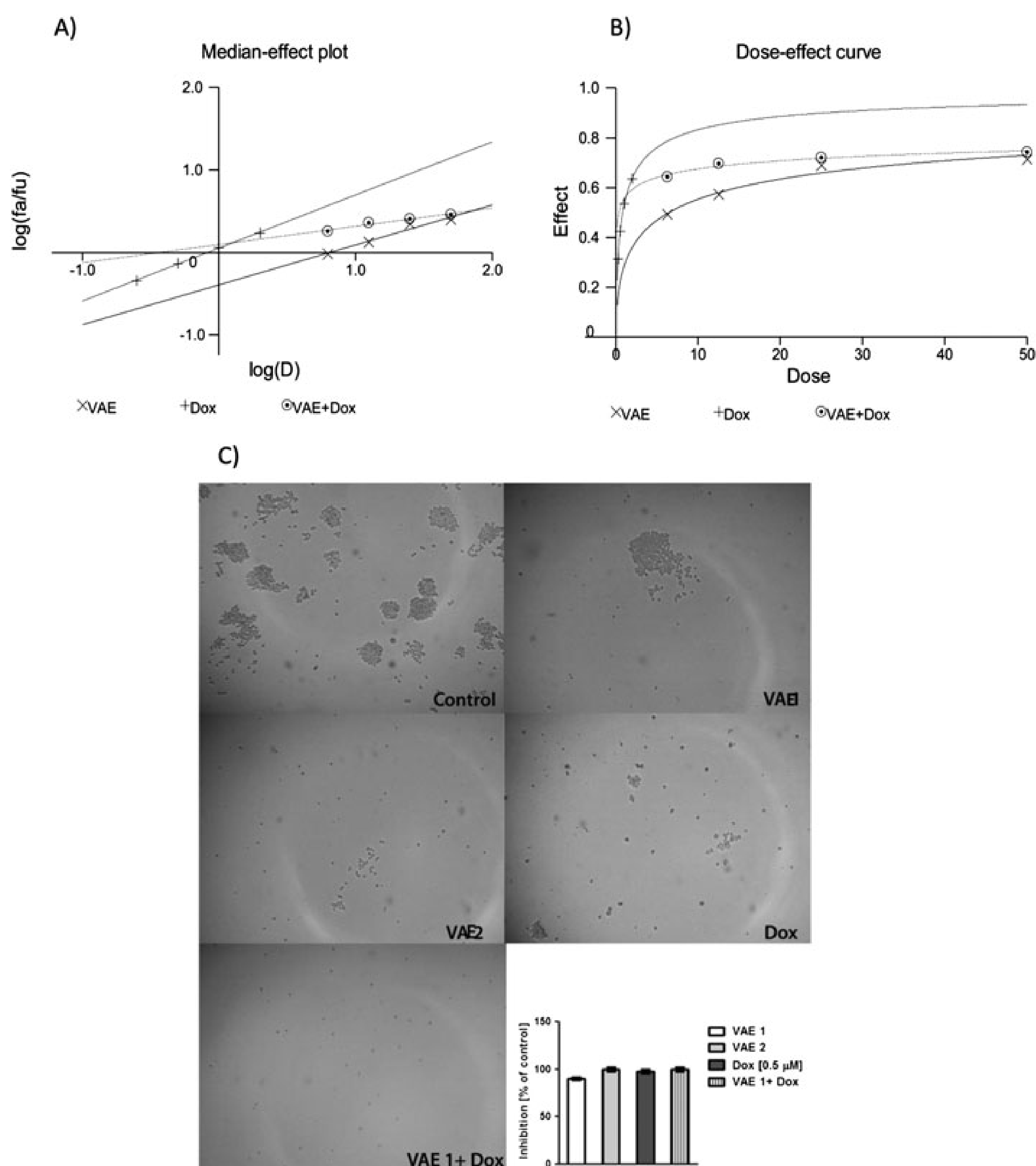

Figure 1. Interaction between mistletoe extract (*Viscum album* (VA) extract) and doxorubicin (Dox). Seventy-two-hour treatment of K562 cells with different concentrations of agents tested: VA extract alone (×); Dox alone (+), or a constant ratio of VA extract and Dox mixture ( ). (A) Median effect plot curve and (B) dose–effect curve represent the data from Table 1. (C) Clonogenicity of K562 cells using sub-therapeutic concentration of Dox (0.5 μM) alone or in combination with two VA extract concentrations: VA 1 = 0.8 μg/mL and VA 2 = 1.6 μg/mL. Figure displays untreated cells (control) and cells incubated for 24 h with investigated compounds. Colonies were scored after 7 days. Experiment was repeated twice using independently derived cell populations and yielded comparable results. A representative experiment is shown.

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Dox has a synergistic inhibitory action in sub-therapeutic Dox concentrations.

Co-treatment of K562 cells with *Viscum album* extract recovers doxorubicin-induced G2/M arrest and reduces expression of cyclins B1 and D1

To address the basis of VAE and Dox co-treatment synergistic action, we have decided to investigate potential changes in K562 cell cycle progression during various treatment conditions. Our dose–effect curve indicates that VAE treatment-induced toxicity above 2.0 µg/mL does not show linear relationship to concentration (Fig. 1B). To account for possible saturation and/or mechanistic crosstalk between high concentration treatments with VAE and Dox that may result in antagonism observed, we have selected 2.0 µg/mL VAE along with 0.5 µM (low) and 2.0 µM (high) Dox concentrations for cell cycle distribution analysis.

Viscum album extract and Dox mono-treatment and co-treatment exhibited time-dependent effects on K562 cell cycle progression and toxicity. Twenty-four-hour and 48-h co-treatment of K562 cells with VAE and Dox compared with Dox alone resulted in a gradual increase of dying cell population residing in the sub-G1 phase and a reduction of cells in both S and G2/M phases of the cell cycle (Figure S1).

Extending K562 cell line treatment to 72 h with VAE in combination with both concentrations of Dox (0.5 and

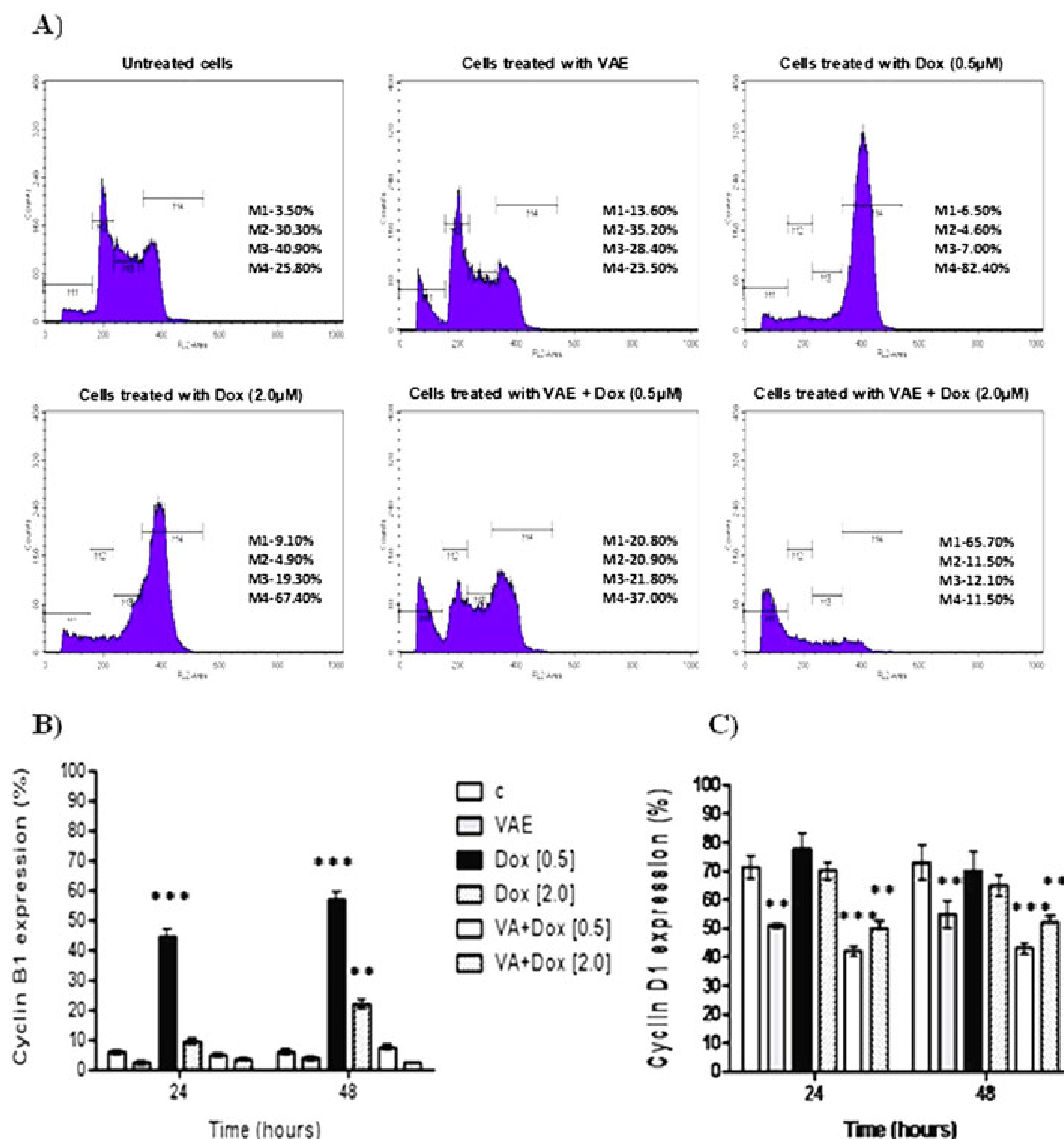

Figure 2. Fluorescence-activated cell sorting analysis of cell cycle distribution and markers of cell cycle arrest in K562-treated leukemia cells. (A) Graphic representation of K562 cell cycle phase distribution upon 72-h treatment with *Viscum album* extract (VAE; 2 µg/mL) and Dox (0.5 and 2 µM) alone or in combination. Cultures were stained with propidium iodide and analyzed for alternations in cell cycle phase distribution by flow cytometry. (B) Expression of cyclin B1 and (C) cyclin D1 upon treatment with indicated concentrations of Dox and VAE for 24 and 48 h. Results are presented as the mean ± standard error of the mean of three independent experiments. Asterisks denote statistical significance compared with control cells (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). M2—Cells with DNA content corresponding to G0/G1 phases. M3—Cells with DNA content corresponding to S phase. M4—Cells with DNA content corresponding to G2/M phases.

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2.0 μM) resulted in further cell death increase (sub-G1 phase cells 20.8% and 65.7%, respectively) and dramatic reduction of G2/M cell population (Fig. 2A).

Next, we performed FACS analysis of time-dependent cell death induction in K562 cells using different treatment combinations (Fig. 3A). Cells were incubated with investigated compounds for 24, 48, and 72 h and stained for expression of early apoptotic marker annexin V and PI permeability (Fig. 3A).

In line with cell cycle phase distribution results, short-term treatment (24 and 48 h) with investigated agents alone did not induce significant increase in programmed cell death (Fig. 3A).

Importantly, extending treatment with VAE and Dox to 72 h resulted in the onset of massive cell death at sub-apoptotic Dox concentrations (0.5 μM), sharply contrasting the effects of VAE and Dox single-treatment effects under the same conditions (Fig. 3A). Early apoptosis-inducing effects were dose and time dependent with the highest number of early apoptotic K562 cells (65%, $p < 0.001$) detected 72 h after co-treatment with 2.0 μg/mL VAE and 2.0 μM Dox (Fig. 3A). Interestingly, while percentage of early apoptotic cell population increases after VAE/Dox co-treatment, late apoptotic and necrotic cell populations are almost lost (annexin V-positive/PI-positive and annexin V-negative/PI-positive cells, respectively) (Fig. 3A), probably suggesting a delay in the activation of apoptotic execution machinery.

The G2/M block induced by treatment of K562 cells with Dox alone was completely recovered after co-treatment with VAE. Moreover, 72 h after co-treatment with VAE and Dox, most cells have accumulated in a sub-G1 phase indicating cell death (Fig. 2A). Previous work shows that p53-deficient cancer cells are resistant to chemotherapeutics (Lowe *et al.*, 1994) and as response to treatment induce G2/M arrest (Meng *et al.*, 2013; Wang *et al.*, 2009). However, sensitivity of cancer cells to Dox depends on their ability to activate S-phase checkpoint controlled by p53 (Siu *et al.*, 1999). To gain insight into events preceding sub-G1 accumulation of p53 null K562 cells, we measured expression of two major S and M phase checkpoint cyclins, cyclin B1 and cyclin D1 during shorter treatments (24 and 48 h) (Figs. 2B and C). Flow cytometric analysis revealed that sub-therapeutic concentrations of Dox (0.5 μM) induce cyclin B1 expression in a time-dependent manner (Fig. 2B). Increased expression of cyclin B1 observed during extended sub-therapeutic Dox treatment was associated with an accumulation of K562 Dox-treated cells in the G2/M phase and was recovered during VAE and Dox treatment reflected by a size reduction of cyclin B1-positive cell population (Fig. 2B). Twenty-four-hour and 48-h treatment of K562 cells with Dox alone revealed no significant differences in the percentage of cyclin D1-positive cells compared with controls (Fig. 2C). Interestingly, incubation of cells with VAE alone or in combination with Dox significantly reduced the percentage of cyclin D1-positive cells at both time points and at both concentrations (Fig. 2C). These results suggest that VAE/Dox co-treatment of K562 leukemia cells bypasses

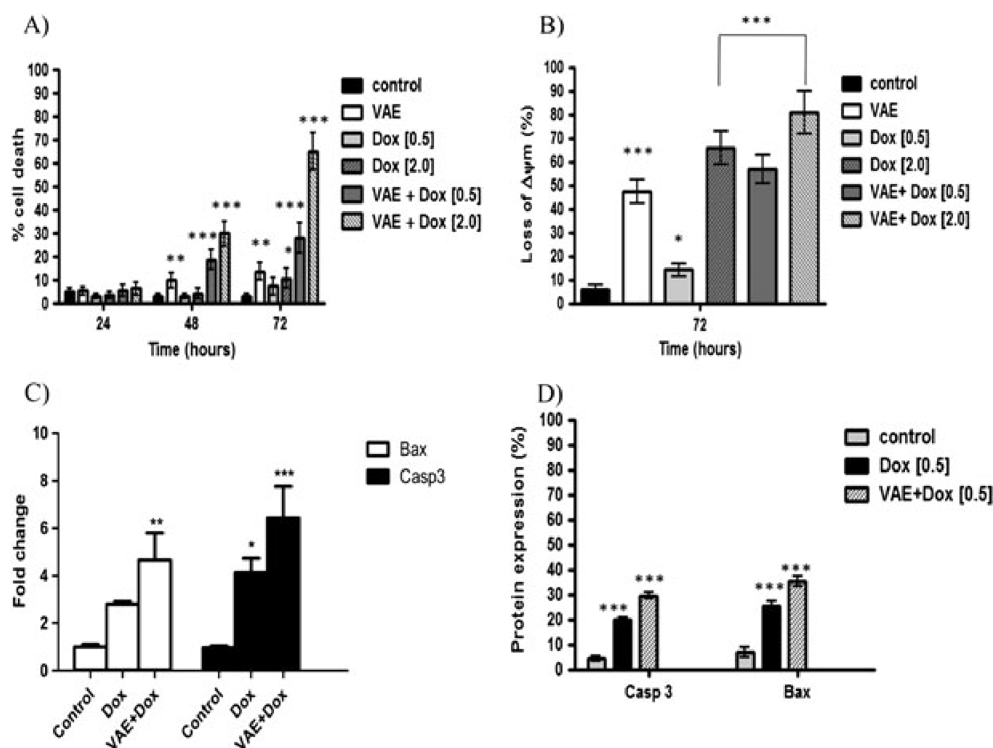

Figure 3. Apoptotic response of K562 doxorubicin (Dox)-resistant cells upon co-treatment with *Viscum album* extract (VAE). Cell were treated with VAE (2.0 μg/mL) and Dox (0.5 and 2 μM) alone or in combination for indicated times. (A) Cell death was measured by the bivariate Annexin V/propidium iodide flow cytometry. (B) Dissipation of mitochondrial membrane potential was assessed by flow cytometry using rhodamine 123 staining. Seventy-two-hour treatment of K562 with VAE (2.0 μg/mL) and sub-apoptotic concentration of Dox (0.5 μM) alone, or in combination, increases caspase 3 and Bax mRNA expression. (C) Resulting and corresponding increase in protein levels. (D) mRNA levels were normalized to GAPDH. All results are presented as a mean ± standard error of the mean of three independent experiments. Asterisks denote statistical significance compared with control cells (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

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Dox-mediated G2/M arrest by modulating expression of cyclins B1 and D1.

Viscum album extract/doxorubicin co-treatment increases apoptotic response in K562 leukemia cells

Our annexin V/PI FACS analysis data revealed increased population of early apoptotic K562 cells upon co-treatment with Dox and VAE (Fig. 3A). Therefore, we sought to assess whether synergistic action of VAE and Dox on K562 leukemia cells results in increased mitochondrial apoptotic pathway activation and intrinsic apoptotic pathway execution. It has been described that cytotoxic drugs activate intrinsic/mitochondrial apoptotic pathway (Fulda and Debatin, 2004) reflected by measurable changes in mitochondrial membrane potential (Δψm) (Decaudin *et al.*, 1998; Green and Kroemer, 2004), release of cytochrome C, proapoptotic factor Bax, and effector caspase 3 activation (Marzo *et al.*, 1998; Petit *et al.*, 1997; Saleh *et al.*, 1999; Shimizu *et al.*, 1999).

We performed FACS Rh123 fluorochrome incorporation assay (Yan *et al.*, 2007). K562 cells were incubated with 2.0 μg/mL of VAE and 0.5 and 2.0 μM Dox for 24, 48, and 72 h. The Δψm did not change significantly after 24 and 48 h of K562 cell treatment with either VAE or Dox treatment, single or in combination (Figure S2). In contrast, a dramatic loss of mitochondrial membrane potential (Δψm) in K562 cells was observed upon 72-h co-treatment with VAE, Dox, and a combination of both (Fig. 3B). Induced expression and translocation of proapoptotic Bax protein to mitochondria results in membrane pore assembly and dissipation of the membrane potential (Marzo *et al.*, 1998). If followed by cytochrome C release to cytosol, it initiates a cascade of events resulting in caspase 9-mediated cleavage and activation of caspase 3, one of the key executioner caspases of the intrinsic apoptotic program. Therefore, we decided to evaluate Bax and caspase 3 expression and activation during VAE and Dox co-treatment at single lower concentrations where the strongest synergistic action was observed (2.0 μg/mL VAE and 0.5 μM Dox, Table 1). As in our previous experiments, cells were treated with VAE (2.0 μg/mL) and Dox (0.5 μM) for the period of 72 h. Quantitative polymerase chain reaction analysis of caspase-3 and Bax gene expression after 72-h co-treatment with VAE and Dox revealed increased expression of both genes compared with mono-treated or untreated controls (Fig. 3C). In line with the mRNA expression data, FACS staining for Bax protein and cleaved caspase-3 showed increased protein expression, confirming activation of the intrinsic apoptotic pathway signaling (Fig. 3D). Data presented here indicate that synergistic antileukemic activity of VAE and Dox co-treatment at sub-therapeutic and sub-apoptotic concentrations is achieved by higher activation and execution of the intrinsic apoptotic program.

DISCUSSION

The goal of cancer therapy is to cure the disease, prevent its recurrence, and decrease mortality. Despite great advancements in the area of individualized medicine, chemotherapeutics often remain a therapy of choice especially for advanced malignancies (Jackson and Chester, 2015). Because of their non-selective mode of action, severe side effects and toxicity still represent a great challenge in oncotherapy (Dicato, 2012). Therefore, identification of medicines that would allow for chemotherapeutic dose reduction, without altering their overall efficacy, remains an important task for cancer researchers today. CML represents 14% of all leukemias diagnosed with median age of patients affected being 60–65 years. Majority of CML patients are successfully treated with *Bcr-Abl* kinase inhibitor imatinib (Valent, 2007). However, patients with blast-phase CML presenting as acute lymphoblastic leukemia will eventually undergo treatment with chemotherapeutics including Dox.

Chronic myelogenous leukemia cells expressing oncogenic tyrosine kinase such as *Bcr-Abl* reveal significant resistance to Dox, warranting use of higher drug concentrations in therapy (Blagosklonny *et al.*, 2001; Fang *et al.*, 2000). In order to reduce the side effects and improve effectiveness of standard chemotherapy, many CAMs have been tested so far. Mistletoe extracts are among the commonly prescribed CAMs for cancer patients.

Previous work has described synergistic interactions between *V. album* extract and chemotherapeutics only at high concentrations of chemotherapeutics (Hong *et al.*, 2014; Siegle *et al.*, 2001; Weissenstein *et al.*, 2014). Furthermore, most studies report synergistic antiproliferative but not apoptotic effects of VAE with low concentrations of chemotherapeutics (Weissenstein *et al.*, 2014).We report that optimal synergistic antitumor activity of Dox and *V. album* aqueous extract obtained by UAE is achieved at sub-apoptotic Dox concentrations accompanied by dissipation of mitochondrial membrane potential l (Δψm) and upregulation of Bax and caspase-3 (Fig. 3), confirming the completion of the apoptotic program. Direct antiproliferative and cytotoxic activity of mistletoe is based mainly on a dose-dependent apoptotic effect of MLs (Janssen *et al.*, 1993). Similar to our results, ML-I antitumor activity was shown to be mediated via 28S ribosomal subunit inactivation, subsequent inhibition of protein synthesis, and induction of intrinsic apoptotic pathway (Bantel *et al.*, 1999; Endo *et al.*, 1988). These paralleling findings may reflect activity of ML-I in our VAE. Treatment of human peripheral blood lymphocytes, human peripheral blood monocytes, murine thymocytes, and human monocytic THP-1 cells with ML-1/VAA-I exerts cytotoxicity in a dose-dependent and time-dependent manner at concentrations above 10 ng/mL with the onset at 24 h (Hostanska *et al.*, 1996–1997). Our results show that 72-h treatment of PBMCs with 2 μg/mL of aqueous VAE obtained by UAE does not exert toxic effects with IC_{50} being 13.6 μg/mL, providing support for testing of this VAE formulation *in vivo* and raising the question of other metabolites that may confer high antitumor action. It has been shown that ML addition to cancer therapy using DNA-damaging agents improves efficacy of DNA repair in PBMCs and overall better therapy tolerance (Burkhart *et al.*, 2010). However, clinical trials have not conclusively demonstrated survival advantage or reduced disease progression upon VAE addition to chemotherapy regimen. These results could be reconciled by the assumption that VAE addition to chemotherapy

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regimen may improve both DNA repair systems in healthy and tumor cells especially at sub-apoptotic doses of chemotherapeutics. Thus, we have employed a UAE protocol and obtained high anticancer activity mistletoe water extract under the predicament that content of active polypeptides such as MLs responsible for putative tumor DNA repair activity and overt PBMC toxicity will be reduced (Jiménez *et al*., 2008; Stathopulos *et al*., 2004).

Also, it has been previously reported that cancer toxicity of aqueous extracts is more potent than single components such as MLs (Eggenschwiler *et al*., 2007). Polyphenols are present in rather high concentrations and exhibit specific content profile in different mistletoe preparations (Schramm, 2015). Polyphenols of mistletoe extracts are attractive targets for oncological research as they exert cytotoxic, antiinflammatory, and antihormonal activity and interfere with tumor metabolism (Schramm, 2015; Urech, 2015) but only in concert with other active metabolites (Sak, 2014). Although flavonoids with described synergistic antitumor activity with Dox such as quercetin (Wang *et al*., 2012) are unlikely to be significantly represented in our preparation, other very polar flavonoids and polyphenolics with as yet uncharacterized anticancer activity are likely represented. It is necessary to determine precise identification and quantification of ML fractions that still retain activity in the extract obtained with this method, as well as activity of flavonoids and other polyphenols that may be responsible for the additive cancer toxicity with low-dose Dox observed.

Prolonged G2/M arrest of chemotherapy-resistant cancer cells is transient and serves to repair DNA damage (Hirose *et al*., 2001a, 2001b; Shah and Schwartz, 2001).

Cancer cell ability to activate checkpoint signaling in response to DNA damage induced by Dox prevents progression of the cell cycle leading to chemoresistance (Meng *et al*., 2013). Conversely, previous reports suggest that abrogation of the G2 checkpoint of p53-deficient cancers results in sensitization to chemotherapy (Hirose *et al*., 2001a, 2001b; Luo *et al*., 2001; Meng *et al*., 2013). Our data reveal that VAE sensitizes K562 cells to low-dose Dox treatment by inhibiting Dox-induced G2/M arrest. While VAE alone exhibits only neglectable toxicity on healthy PBMCs (IC_{50} value at 13.36 μg/mL), co-treatment with low-dose Dox (0.5 μM) enhances K562 apoptosis providing effectiveness comparable with higher Dox concentrations (2 μM) (Table 1, Figs. 2A and 3A, B). Furthermore, our results suggest that the synergistic toxicity of VAE with Dox relies upon abrogation of Dox-induced G2/M arrest (Fig. 2A). G2 checkpoint is maintained by Chk1, Chk2, p53, and MK2-mediated inhibition of CDC25 phosphatases that act as activators of cyclin B/Cdc2 promoting mitosis (Boutros *et al*., 2007; Donzelli and Draetta, 2003). During G1-phase progression, cyclin D-Cdk4, cyclin D-Cdk6, and cyclin E-Cdk2 complexes are activated, controlling G1/S transition (Quelle *et al*., 1993). Recent findings suggest that Dox-induced cyclin D1 expression in p53-deficient HC-60 cells may be responsible for treatment survival and perturbations of G1 and G2 checkpoints observed (Zuryn *et al*., 2012, 2014). On the other hand, cyclin B1 and cdc2 (Cdk1) form a complex named ‘mitotic promoting factor’ (MPF) crucial for G2/M transition (Pines and Hunter, 1990). In this paper, we show that VAE treatment effectively inhibits expression of cyclin D1 in K562 cells alone or in combination with Dox (Fig. 2C). However, treatment of K562 cells with Dox alone did not reveal statistically significant difference in percentage of cyclin D1-positive cells compared with controls (Fig. 2C). Absence of Dox treatment effect on cyclin D1 expression may be attributed to relatively high resistance of K562 cells to Dox but also cell type-specific mechanisms being active in different p53 null cancers. Dan and Yamori (2001) suggested that changes in the level of cyclin B1 and G2/M arrest in A549 cells exposed to Dox are regulated in the manner independent of p53 because similar changes were not induced by cisplatin, a well-known p53 activator. In agreement with these results, it was shown that an increased level of cyclin B1 may appear in some G2/M-arrested cancer cells with p53 deficiency such as K562 cells (Cerquetti *et al*., 2008). Moreover, activation of antiapoptotic proteins such as Bcl-xL and G2/M arrest have been proposed to represent a crucial event conferring drug resistance in p53-deficient Bcr-abl-expressing cancer cells (Amarante-Mendes *et al*., 1998a; Bedi *et al*., 1995). Interestingly, expression levels of cyclin B1 seem to be increased only during low-dose Dox treatment (Fig. 2B) and are markedly reduced by addition of VAE (Fig. 2B). Such parallelism of endogenous expression changes observed for cyclins B1 and D1 during co-treatment with low-dose Dox (0.5 μM) and VAE may indeed reflect their requirement for G2/M arrest observed in our experiments.

In summary, our results indicate that VAE sensitizes *Bcr-Abl*-positive K562 cells to low-dose Dox treatment (0.5 μM) by abrogating G2/M accumulation and inducing apoptosis comparable with highly toxic Dox concentrations (2 μM). These effects may be essential for damaged *Bcr-Abl*-positive cell recovery upon treatment with sub-apoptotic concentrations of Dox and selection of clones resistant to genotoxic treatment. Further research focusing on identification of VAE anticancer compounds that would allow for standardized treatment with chemotherapeutics in cancer therapy is warranted.

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AUTHOR’S CONTRIBUTIONS

T. S-R., N. T-M., and M. C. designed the study, performed the experiments, analyzed and interpreted the data, and drafted the manuscript. K. K. and A. K-R. participated in the design of the experiments, revised critically the manuscript, and contributed to the discussion. D. G. revised critically the manuscript and added important points to the discussion. K. S. prepared plant extract, revised critically the manuscript, and added important points to the discussion. T. Z. designed the study, analyzed and interpreted the data, and wrote the manuscript. All the authors approved the final draft of the manuscript.

Conflict of Interest

The authors declare that there are no conflicts of interests.

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REFERENCES

Amarante-Mendes GP, McGahon AJ, Nishioka WK, Afar DE, Witte ON, Green DR. 1998a. Bcl-2-independent Bcr-Abl-mediated resistance to apoptosis: protection is correlated with up regulation of Bcl-xL. *Oncogene* **16**: 1383–1390.

Amer B, Juvika OJ, Dupontb F, Francisa GW, Fossena T. 2012. Novel aminoalkaloids from European mistletoe (*Viscum album* L.). *Phytochem Lett* **5**: 677–681.

Arda N, Onay E, Koz O, Kirmizigul S. 2003. Monosaccharides and polyols from mistletoes (*Viscum album* L.) growing on two different host species. *Biologia (Bratislava)* **58**: 1037–1041.

Augustin M, Bock PR, Hanisch J, Karasmann M, Schneider B. 2005. Safety and efficacy of the long-term adjuvant treatment of primary intermediate- to high-risk malignant melanoma (UICC/AJCC stage II and III) with a standardized fermented European mistletoe (*Viscum album* L.) extract. Results from a multicenter, comparative, epidemiological cohort study in Germany and Switzerland. *Arzneimittelforschung* **55**: 38–49.

Bantel H, Engels IH, Voelter W, Schulze-Osthoff K, Wesselborg S. 1999. Mistletoe lectin activates caspase-8/FLICE independently of death receptor signaling and enhances anticancer drug-induced apoptosis. *Cancer Res* **59**: 2083–2090.

Bedi A, Barber JP, Bedi GC, *et al*. 1995. BCR-ABL-mediated inhibition of apoptosis with delay of G2/M transition after DNA damage: a mechanism of resistance to multiple anticancer agents. *Blood* **86**: 1148–1158.

Beuth J, Schierholz JM. 2007. Evidence-based complementary oncology. Innovative approaches to optimize standard therapy strategies. *In Vivo* **21**: 423–428.

Blagosklonny MV, Fojo T, Bhalla KN, *et al*. 2001. The Hsp90 inhibitor geldanamycin selectively sensitizes Bcr-Abl-expressing leukemia cells to cytotoxic chemotherapy. *Leukemia* **15**: 1537–1543.

Boutros R, Lobjois V, Ducommun B. 2007. CDC25 phosphatases in cancer cells: key players? Good targets? *Nat Rev Cancer* **7**: 495–507.

Burkhart J, Wälchli C, Heusser P, Weissenstein U, Baumgartner S, Andres AC. 2010. *In vitro* investigation into the potential of a mistletoe extract to alleviate adverse effects of cyclophosphamide. *Altern Ther Health Med* **16**: 40–48.

Büssing A. 2000. Mistletoe: The Genus *Viscum*. Hardwood Academic Publishers: Amsterdam.

Bussing A, Schietzel M. 1999. Apoptosis-inducing properties of *Viscum album* L. extracts from different host trees, correlate with their content of toxic mistletoe lectins. *Anticancer Res* **19**: 23–28.

Bussing A, Bischof M, Hatzmann W, *et al*. 2005. Prevention of surgery-induced suppression of granulocyte function by intravenous application of a fermented extract from *Viscum album* L. in breast cancer patients. *Anticancer Res* **25**: 4753–4757.

Cebović T, Spasić S, Popović M. 2008. Cytotoxic effects of the *Viscum album* L. extract on Ehrlich tumour cells *in vivo*. *Phytother Res* **22**: 1097–1103.

Cerquetti L, Bucci B, Marchese R, *et al*. 2008. Mitotane increases the radiotherapy inhibitory effect and induces G2-arrest in combined treatment on both H295R and SW13 adrenocortical cell lines. *Endocr Relat Cancer* **15**: 623–634.

Chou TC, Talalay P. 1984. Quantitative analysis of dose–effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul* **22**: 27–55.

Cordero CM, Gil Serrano AM, Ayuso Gonzalez MJ. 1993. Transfer of bipiperidyl and quinolizidine alkaloids to *Viscum cruciatum* Sieber (Loranthaceae) hemiparasitic on *Retama sphaerocarpa* Boissier (Leguminosae). *J Chem Ecol* **19**: 2389–2393.

Daley GQ, Baltimore D. 1988. Transformation of an interleukin 3-dependent hematopoietic cell line by the chronic myelogenous leukemia-specific P210bcr/abl protein. *Proc Natl Acad Sci U S A* **85**: 9312–9316.

Dan S, Yamori T. 2001. Repression of cyclin B1 expression after treatment with adriamycin, but not cisplatin in human lung cancer A549 cells. *Biochem Biophys Res Commun* **280**: 861–867.

Decaudin D, Marzo I, Brenner C, Kroemer G. 1998. Mitochondria in chemotherapy-induced apoptosis: a prospective novel target of cancer therapy (review). *Int J Oncol* **12**: 141–152.

Deliorman O, Çalış I, Ergun F. 2002. Two new flavonoid glycosides from *Viscum album* L. ssp. *Album Pharm Biol* **40**: 380–383.

Dewanto V, Wu X, Adom KK, Liu RH. 2002. Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. *J Agric Food Chem* **50**: 3010–3014.

Dicato MA. 2012. Side effects of medical cancer therapy. In Prevention and Treatment. Springer-Verlag: London.

Donzelli M, Draetta GF. 2003. Regulating mammalian checkpoints through Cdc25 inactivation. *EMBO Rep* **4**: 671–677.

Duong Van Huyen JP *et al.* 2002. Induction of apoptosis of endothelial cells by *Viscum album*: a role for anti-tumoral properties of mistletoe lectins. *Mol Med* **8**: 600–606.

Eggenschwiler J, von Balthazar L, Stritt B, *et al*. 2007. Mistletoe lectin is not the only cytotoxic component in fermented preparations of *Viscum album* from white fir (*Abies pectinata*). *BMC Complement Altern Med* **7**: 1–7.

Elsasser-Beile U, Lusebrink S, Grussenmeyer T, Wetterauer U, Schultze-Seemann W. 1998. Comparison of the effects of various clinically applied mistletoe preparations on peripheral blood leukocytes. *Arzneimittelforschung* **48**: 1185–1189.

Endo Y, Tsurugi K, Franz H. 1988. The site of action of the A-chain of mistletoe lectin I on eukaryotic ribosomes. The RNA *N*-glycosidase activity of the protein. *FEBS Lett* **231**: 378–380.

Eshkourfu R, Cobeljic B, Vujcic M, *et al*. 2011. Synthesis, characterization, cytotoxic activity and DNA binding properties of the novel dinuclear cobalt(III) complex with the condensation product of 2-acetylpyridine and malonic acid dihydrazide. *J Inorg Biochem* **105**: 1196–1203.

Fang G, Kim CN, Perkins CL, *et al*. 2000. CGP57148B (STI-571) induces differentiation and apoptosis and sensitizes Bcr-Abl-positive human leukemia cells to apoptosis due to antileukemic drugs. *Blood* **96**: 2246–2253.

Fasching PA, Thiel F, Nicolaisen-Murmann K, *et al*. 2007. Association of complementary methods with quality of life and life satisfaction in patients with gynecologic and breast malignancies. *Support Care Cancer* **15**: 1277–1284.

Fukunaga T, Kajikawa I, Nishiya K, Watanabe Y, Takeya K, Itokawa H. 1987. Studies on the constituents of the European mistletoe, *Viscum album* L. *Chem Pharm Bull* **35**: 3292–3297.

Fulda S, Debatin KM. 2004. Targeting apoptosis pathways in cancer therapy. *Curr Cancer Drug Targets* **4**: 569–576.

Giudici AM, Regente MC, Villalaín J, Pfüller K, Pfüller U, De La Canal L. 2004. Mistletoe viscotoxins induce membrane permeabilization and spore death in phytopathogenic fungi. *Physiol Plant* **121**: 2–7.

Green DR, Kroemer G. 2004. The pathophysiology of mitochondrial cell death. *Science* **305**: 626–629.

Hegnauer R. 1966. Aucuba type glucosides. On their distribution and importance as systemic characteristics. *Pharm Acta Helv* **41**: 577–587.

Hirose Y, Berger MS, Pieper RO. 2001a. Abrogation of the Chk1-mediated G(2) checkpoint pathway potentiates temozolomide-induced toxicity in a p53-independent manner in human glioblastoma cells. *Cancer Res* **61**: 5843–5849.

Hirose Y, Berger MS, Pieper RO. 2001b. p53 effects both the duration of G2/M arrest and the fate of temozolomide-treated human glioblastoma cells. *Cancer Res* **61**: 1957–1963.

Hong CE, Park AK, Lyu SY. 2014. Synergistic anticancer effects of lectin and doxorubicin in breast cancer cells. *Mol Cell Biochem* **394**: 225–235.

Hostanska K, Hajto T, Weber K, *et al*. 1996–1997. A natural immunity-activating plant lectin, *Viscum album* agglutinin-I, induces apoptosis in human lymphocytes, monocytes, monocytic THP-1 cells and murine thymocytes. *Nat Immun* **15**: 295–311.

Jackson SE, Chester JD. 2015. Personalised cancer medicine. *Int J Cancer* **137**: 262–266.

Janssen O, Scheffler A, Kabelitz D. 1993. *In vitro* effects of mistletoe extracts and mistletoe lectins. Cytotoxicity towards tumor cells due to the induction or programmed cell death (apoptosis). *Arzneim-Forsch/Drug Res* **43**: 1221–1227.

Jiménez M, André S, Barillari C, *et al*. 2008. Domain versatility in plant AB-toxins: evidence for a local, pH-dependent rearrangement in the 2gamma lectin site of the mistletoe lectin by applying ligand derivatives and modelling. *FEBS Lett* **582**: 2309–2312.

Johansson B, Fioretos T, Mitelman F. 2002. Cytogenetic and molecular genetic evolution of chronic myeloid leukemia. *Acta Haematol* **107**: 76–94.

Jordan E, Wagner H. 1986. Structure and properties of polysaccharides from *Viscum album* (L.). *Oncology* **43**(Suppl 1): 8–15.

Kienle GS, Kiene H. 2003. Die Mistel in der Onkologie—Fakten und konzeptionelle Grundlagen. Schattauer Verlag: Stuttgart-New York.

Copyright © 2015 John Wiley & Sons, Ltd.

Kienle GS, Kiene H. 2010. Review article: influence of *Viscum album* L (European mistletoe) extracts on quality of life in cancer patients: a systematic review of controlled clinical studies. *Integr Cancer Ther* **9**: 142–157.
Kienle GS, Berrino F, Bussing A, Portalupi E, Rosenzweig S, Kiene H. 2003. Mistletoe in cancer—a systematic review on controlled clinical trials. *Eur J Med Res* **8**: 109–119.
Klopp R, Schmidt W, Werner E, Werner M, Niemer W, Beuth J. 2005. Influence of complementary *Viscum album* (Iscador) administration on microcirculation and immune system of ear, nose and throat carcinoma patients treated with radiation and chemotherapy. *Anticancer Res* **25**: 601–610.
Kruger NJ. 1994. The Bradford method for protein quantitation. In Methods in Molecular Biology. Basic Protein and Peptide Protocols, Walker JM (ed.). Humana Press, Totowa, New Jersey.
Lowe SW, Bodis S, McClatchey A, *et al*. 1994. p53 status and the efficacy of cancer therapy *in vivo*. *Science* **266**: 807–810.
Luczkiewicz M, Cisowski W, Kaiser P, Ochocka R, Piotrowski A. 2001. Comparative analysis of phenolic acids in mistletoe plants from various hosts. *Acta Pol Pharm* **58**: 373–379.
Lugo TG, Pendergast AM, Muller AJ, Witte ON. 1990. Tyrosine kinase activity and transformation potency of Bcr-abl oncogene products. *Science* **247**: 1079–1082.
Luo Y, Rockow-Magnone SK, Joseph MK, *et al*. 2001. Abrogation of G2 checkpoint specifically sensitize p53 defective cells to cancer chemotherapeutic agents. *Anticancer Res* **21**: 23–28.
Marquette C, Nabell L. 2012. Chemotherapy-resistant metastatic breast cancer. *Curr Treat Options Oncol* **13**: 263–275.
Martin HL, Smith L, Tomlinson DC. 2014. Multidrug-resistant breast cancer: current perspectives. In Breast Cancer **6**. Dove Med Press; 1–13.
Marvibaigi M, Supriyanto E, Amini N, Abdul Majid FA, Jaganathan SK. 2014. Preclinical and clinical effects of mistletoe against breast cancer. *Biomed Res Int* **2014**: 785479.
Marzo I, Brenner C, Zamzami N, *et al*. 1998. Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. *Science* **281**: 2027–2031.
Melzer J, Iten F, Hostanska K, Saller R. 2009. Efficacy and safety of mistletoe preparations (*Viscum album*) for patients with cancer diseases. A systematic review. *Forsch Komplementmed* **16**: 217–226.
Meng X, Dizon DS, Yang S, *et al*. 2013. Strategies for molecularly enhanced chemotherapy to achieve synthetic lethality in endometrial tumors with mutant p53. *Obstet Gynecol Int* **2013**: 828165.
Molassiotis A, Fernadez-Ortega P, Pud D, *et al*. 2005. Use of complementary and alternative medicine in cancer patients: a European survey. *Ann Oncol* **16**: 655–663.
Molassiotis A, Browall M, Milovics L, Panteli V, Patiraki E, Fernandez-Ortega P. 2006a. Complementary and alternative medicine use in patients with gynecological cancers in Europe. *Int J Gynecol Cancer* **16**(Suppl 1): 219–224.
Molassiotis A, Scott JA, Kearney N, *et al*. 2006b. Complementary and alternative medicine use in breast cancer patients in Europe. *Support Care Cancer* **14**: 260–267.
Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* **65**: 55–63.
Ohno M, Abe T. 1991. Rapid colorimetric assay for the quantification of leukemia inhibitory factor (LIF) and interleukin-6 (IL-6). *J Immunol Methods* **145**: 199–203.
Ostermann T, Raak C, Bussing A. 2009. Survival of cancer patients treated with mistletoe extract (Iscador): a systematic literature review. *BMC Cancer* **9**: 451.
Panossian A, Kocharian A, Matinian K, *et al*. 1998. Pharmacological activity of phenylpropanoids of the mistletoe, *Viscum album* L., host: *Pyrus caucasica* Fed. *Phytomedicine* **5**: 11–17.
Park WB, Lyu SY, Kim JH, *et al*. 2001. Inhibition of tumor growth and metastasis by Korean mistletoe lectin is associated with apoptosis and antiangiogenesis. *Cancer Biother Radiopharm* **16**: 439–447.
Petit PX, Zamzami N, Vayssiere JL, Mignotte B, Kroemer G, Castedo M. 1997. Implication of mitochondria in apoptosis. *Mol Cell Biochem* **174**: 185–188.
Pines J, Hunter T. 1990. Human cyclin A is adenovirus E1A-associated protein p60 and behaves differently from cyclin B. *Nature* **346**: 760–763.
Quelle DE, Ashmun RA, Shurtleff SA, *et al*. 1993. Overexpression of mouse D-type cyclins accelerates G1 phase in rodent fibroblasts. *Genes Dev* **7**: 1559–1571.
Sak K. 2014. Cytotoxicity of dietary flavonoids on different human cancer types. *Pharmacogn Rev* **8**: 122–146.
Saleh A, Srinivasula SM, Acharya S, Fishel R, Alnemri ES. 1999. Cytochrome c and dATP-mediated oligomerization of Apaf-1 is a prerequisite for procaspase-9 activation. *J Biol Chem* **274**: 17941–17945.
Sattler M, Salgia R. 1997. Activation of hematopoietic growth factor signal transduction pathways by the human oncogene BCR/ABL. *Cytokine Growth Factor Rev* **8**: 63–79.
Schaller G, Urech K, Grazi G, Giannattasio M. 1998. Viscotoxin composition of the three European subspecies of Viscum album. *Planta Med* **64**: 677–678.
Schink M, Troger W, Dabidian A, *et al*. 2007. Mistletoe extract reduces the surgical suppression of natural killer cell activity in cancer patients. A randomized phase III trial. *Forsch Komplementmed* **14**: 9–17.
Schramm HM. 2015. The anti-cancer activity of mistletoe preparations, as related to their polyphenolic profiles. In Translational Research in Biomedicine, vol. **4**, Zänker KS, Kaveri SV (ed). Karger AG: Basel, 24–39.
Shah MA, Schwartz GK. 2001. Cell cycle-mediated drug resistance: an emerging concept in cancer therapy. *Clin Cancer Res* **7**: 2168–2181.
Shimizu S, Narita M, Tsujimoto Y. 1999. Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. *Nature* **399**: 483–487.
Siegle I, Fritz P, McClellan M, Gutzeit S, Murdter TE. 2001. Combined cytotoxic action of *Viscum album* agglutinin-1 and anticancer agents against human A549 lung cancer cells. *Anticancer Res* **21**: 2687–2691.
Siu WY, Yam CH, Poon RY. 1999. G1 versus G2 cell cycle arrest after adriamycin-induced damage in mouse Swiss3T3 cells. *FEBS Lett* **461**: 299–305.
Son GS, Ryu WS, Kim HY, Woo SU, Park KH, Bae JW. 2010. Immunologic response to mistletoe extract (*Viscum album* L.) after conventional treatment in patients with operable breast cancer. *J Breast Canc* **13**: 14–18.
Stathopulos PB, Scholz GA, Hwang YM, Rumfeldt JA, Lepock JR, Meiering EM. 2004. Sonication of proteins causes formation of aggregates that resemble amyloid. *Protein Sci* **13**: 3017–3027.
Troger W, Jezdic S, Zdrale Z, Tisma N, Hamre HJ, Matijasevic M. 2009. Quality of life and neutropenia in patients with early stage breast cancer: a randomized pilot study comparing additional treatment with mistletoe extract to chemotherapy alone. *Breast Cancer (Auckl)* **3**: 35–45.
Troger W, Galun D, Reif M, Schumann A, Stankovic N, Milicevic M. 2013. *Viscum album* [L.] extract therapy in patients with locally advanced or metastatic pancreatic cancer: a randomised clinical trial on overall survival. *Eur J Cancer* **49**: 3788–3797.
Troger W, Galun D, Reif M, Schumann A, Stankovic N, Milicevic M. 2014a. Quality of life of patients with advanced pancreatic cancer during treatment with mistletoe: a randomized controlled trial. *Dtsch Arztebl Int* **111**: 493–502, 433 p following 502.
Troger W, Zdrale Z, Tisma N, Matijasevic M. 2014b. Additional therapy with a mistletoe product during adjuvant chemotherapy of breast cancer patients improves quality of life: an open randomized clinical pilot trial. *Evid Based Complement Alternat Med* **2014**: 430518.
Urech K. 2015. Chemical constituents of *Viscum album* L.: implications for the pharmaceutical preparation of mistletoe. In Translation Research in Biomedicine, Zänker KS, Kaveri SV (ed). Karger AG: Basel, vol. **4**, 11–24
Valent P. 2007. Imatinib-resistant chronic myeloid leukemia (CML): current concepts on pathogenesis and new emerging pharmacologic approaches. *Biologics* **1**: 433–448.
Valentiner U, Pfuller U, Baum C, Schumacher U. 2002. The cytotoxic effect of mistletoe lectins I, II and III on sensitive and multidrug resistant human colon cancer cell lines *in vitro*. *Toxicology* **171**: 187–199.
Vicas S, Rugină D, Leopold L, Pintea A, Socaciu C. 2011. HPLC fingerprint of bioactive compounds and antioxidant activities of *Viscum album* from different host trees. *Not Bot Horti Agrobot Cluj Napoca* **39**: 48–57.
Wacker R, Stoeva S, Pfüller K, Pfüller U, Voelter W. 2004. Complete structure determination of the A chain of mistletoe lectin III from *Viscum album* L. ssp. *album*. *J Pept Sci* **10**: 138–148.
Wagner H, Jordan E, Feil B. 1986. Studies on the standardization of mistletoe preparations. *Oncology* **43**(Suppl): 16–22.

Copyright © 2015 John Wiley & Sons, Ltd.

Wang Y, Ji P, Liu J, Broaddus RR, Xue F, Zhang W. 2009. Centrosome-associated regulators of the G(2)/M checkpoint as targets for cancer therapy. *Mol Cancer* **8**: 8.

Wang G, Zhang J, Liu L, Sharma S, Dong Q. 2012. Quercetin potentiates doxorubicin mediated antitumor effects against liver cancer through p53/Bcl-xl. *PLoS One* **7**: e51764.

Weissenstein U, Kunz M, Urech K, Baumgartner S. 2014. Interaction of standardized mistletoe (*Viscum album*) extracts with chemotherapeutic drugs regarding cytostatic and cytotoxic effects *in vitro*. *BMC Complement Altern Med* **8**: 14–16.

Wolfe K, Wu X, Liu RH. 2003. Antioxidant activity of apple peels. *J Agric Food Chem* **51**: 609–614.

Yan M, Zhu P, Liu HM, Zhang HT, Liu L. 2007. Ethanol induced mitochondria injury and permeability transition pore opening: role of mitochondria in alcoholic liver disease. *World J Gastroenterol* **13**: 2352–2356.

Zuryn A, Litwiniec A, Gackowska L, Pawlik A, Grzanka AA, Grzanka A. 2012. Expression of cyclin A, B1 and D1 after induction of cell cycle arrest in the Jurkat cell line exposed to doxorubicin. *Cell Biol Int* **36**: 1129–1135.

Zuryn A, Litwiniec A, Klimaszewska-Wisniewska A, *et al*. 2014. Expression of cyclin D1 after treatment with doxorubicin in the HL-60 cell line. *Cell Biol Int* **38**: 857–867.

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