www.elsevier.com/locate/jep

(3'*R*)-hydroxytabernaelegantine C: a bisindole alkaloid with potent apoptosis inducing activity in colon (HCT116, SW620) and liver (HepG2) cancer cells

Angela Paterna, Sofia E. Gomes, Pedro M. Borralho, Silva Mulhovo, Cecília M.P. Rodrigues, Maria-José U. Ferreira

PII: S0378-8741(16)30742-5
DOI: http://dx.doi.org/10.1016/j.jep.2016.09.020
Reference: JEP10420

To appear in: *Journal of Ethnopharmacology*

Received date: 11 April 2016
Revised date: 1 September 2016
Accepted date: 7 September 2016

Cite this article as: Angela Paterna, Sofia E. Gomes, Pedro M. Borralho, Silva Mulhovo, Cecília M.P. Rodrigues and Maria-José U. Ferreira, (3'*R*) hydroxytabernaelegantine C: a bisindole alkaloid with potent apoptosis inducing activity in colon (HCT116, SW620) and liver (HepG2) cancer cells, *Journal of Ethnopharmacology,* http://dx.doi.org/10.1016/j.jep.2016.09.020

(3'*R*)-hydroxytabernaelegantine C: a bisindole alkaloid with potent apoptosis inducing activity in colon (HCT116, SW620) and liver (HepG2) cancer cells

Angela Paterna[a1], Sofia E. Gomes[a1], Pedro M. Borralho[a], Silva Mulhovo[b], Cecília M. P. Rodrigues[a], Maria-José U. Ferreira[a*]

[a]Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, Universidade de Lisboa, Av. Prof. Gama Pinto, 1649-003 Lisboa, Portugal

[b]Centro de Estudos Moçambicanos e de Etnociências (CEMEC), Faculty of Natural Sciences and Mathematics, Pedagogical University, 21402161 Maputo, Mozambique

apaterna@ff.ulisboa.pt
segomes@ff.ulisboa.pt
borralho@ff.ulisboa.pt
smulhovo@hotmail.com
cmprodrigues@ff.ul.pt
[*]**Corresponding author**. Tel.: +351 21 7946475; fax: +351 21 7946470. mjuferreira@ff.ul.pt

Aut**hor Contributions**

All authors contributed equally to this work.

Abstract

Ethnopharmacological relevance

Tabernaemontana elegans Stapf. (Apocynaceae) is a medicinal plant traditionally used in African countries to treat cancer.

Aims of the study

[1] First co-authors

To discover new apoptosis inducing lead compounds from *T. elegans* and provide scientific validation of the ethnopharmacological use of this plant.

Materials and methods

Through fractionation, (3'R)-hydroxytaberanelegantine C (**1**), a vobasinyl-iboga bisindole alkaloid, was isolated from a cytotoxic alkaloid fraction of the methanol extract of *T. elegans* roots. Its structure was identified by spectroscopic methods, mainly 1D and 2D NMR experiments. Compound **1** was evaluated for its ability to induce apoptosis in HCT116 and SW620 colon and HepG2 liver carcinoma cells. The cell viability of compound **1** was evaluated by the MTS and lactate dehydrogenase (LDH) assays. Induction of apoptosis was analyzed through Guava ViaCount assay, by flow cytometry, caspase-3/7 activity assays and evaluation of nuclear morphology by Hoechst staining. To determine the molecular pathways elicited by **1** exposure, immunoblot analysis was also performed.

Results

(3'*R*)-hydroxytaberanelegantine C (**1**) displayed strong apoptosis induction activity as compared to 5-fluorouracil (5-FU), the most used anticancer agent in colorectal cancer treatment. In the MTS assay, compound **1** exhibited IC_{50} values similar or lower than 5-FU in the three cell lines tested. The IC_{50} value of **1** was also calculated in CCD18co normal human colon fibroblasts. The lactate dehydrogenase assay showed increased LDH release by compound **1**, and the Guava ViaCount assay revealed that **1** significantly increased the incidence of apoptosis to a further extent than 5-FU. Moreover, the induction of apoptosis was corroborated by evaluation of nuclear morphology by Hoechst staining and caspase-3/7 activity assays of **1** treated cells. As expected, in immunoblot analysis, compound **1** treatment led to poly(ADP-ribose) polymerase cleavage. This was

accompanied by decreased anti-apoptotic proteins Bcl-2 and XIAP steady state levels in all three cancer cell lines tested.

Conclusions

Compound **1** showed remarkable induction of apoptosis in HCT116, SW620 and HepG2 cells. Together, the results suggest that compound **1** is a promising lead structure for inducing apoptosis.

Keywords*:* Apocynaceae; *Tabernaemontana elegans*; bisindole alkaloid; anticancer; apoptosis induction activity.

1. Introduction

Cancer is one of the leading causes of morbidity and mortality worldwide (Ferlay et al., 2015). The main challenge in anticancer drug treatment is multidrug resistance (MDR) (Baird and Kaye, 2003; Lage, 2008). There are several mechanisms of MDR to anticancer drugs, such as evasion of drug-induced apoptosis (Gottesman et al., 2002). Deregulation of apoptosis, a regulated form of cell death, may contribute to carcinogenesis, tumor progression and also to confer drug resistance (Baird and Kaye, 2003; Lage, 2008). Apoptosis plays a crucial role in the regulation of many normal physiological and pathophysiological processes, involving a series of biochemical events that lead to characteristic morphologic cell changes, and cell demise. These changes include membrane blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation, finally leading to the death of the cell. Deregulation of apoptosis pathways cause imbalance between cell proliferation and cell death, contributing to several diseases, including cancer (Elmore, 2007; Fulda, 2015). Therapeutic targeting of apoptosis is thus recognized to be promising in anticancer

drug development and a valuable strategy for overcoming MDR (Cragg and Newman, 2005; Mishra and Tiwari, 2011).

Plant-derived compounds remain an important source in the development of new drugs (Bhanot et al., 2011; Newman and Cragg, 2012), being most of the current anticancer drugs from natural origin, particularly from plants (Cragg et al., 2009).

Tabernaemontana species (Apocynaceae), found in tropical and subtropical regions of the world including Africa, are medicinal plants known for producing large amounts and wide variety of bioactive indole and bisindole alkaloids of diverse and complex skeletal types (Vanbeek et al., 1984; Leonard, 1999). The wide range of biological activities of indole alkaloids include cytotoxic and anti-inflammatory properties (Carbone et al., 2015, 2013; de Sa et al., 2009; Ishikura et al., 2013). Vincristine and vinblastine, clinically important anti-cancer agents, are examples of useful bioactive indole alkaloid from Apocynaceae (Cragg and Newman, 2005). Several studies reported the use of *Tabernaemontana* species in African traditional medicine to cure various diseases. In particular, *Tabernaemontana elegans* Stapf. roots bark are used to treat cancer (Vanbeek et al. 1984; Chhabra et al., 1987; Shmelzer and Gurib-Fakim, 2008).

Our ongoing search on new effective anticancer compounds from plants (Duarte et al., 2007; Ferreira et al., 2014; Mansoor et al. 2012, 2011; Matos et al., 2015; Reis et al., 2013) conduct us to the phytochemical study of the African medicinal plant *T. elegans*. Investigation of the alkaloid fraction of the methanol extract of *T. elegans* roots led to the isolation of the monoterpene indole alkaloids dregamine, tabernaemontanine, 16-epidregamine, voacangine, vobasine, and the bisindoles tabernaelegantine C and tabernaelegantinine B. Among these alkaloids, both bisindoles have shown apoptosis inducing activity in HCT116 colon cancer cells (Mansoor et al., 2013). Three hemi-synthetic hydrazone derivatives of dregamine and tabernaemontanine, two inactive

epimers isolated in large amounts, have also exhibited significant pro-apoptotic activity (Paterna et al., 2015).

In the present study, aiming to find new apoptosis inducing lead compounds, we have isolated a vobasinyl-iboga bisindole alkaloid (**1**) from the cytotoxic alkaloid fraction of the methanol extract of *T. elegans roots*, which was evaluated for its ability to induce apoptosis in HCT116 and SW620 colon and HepG2 liver carcinoma cells. To further explore the molecular pathways by which **1** exerts its cytotoxic and pro-apoptotic effect, immunoblot analysis of key apoptosis regulators was performed, using total protein extract from HCT116, SW620 or HepG2 cells.

2. Material and methods

2.1. General experimental procedures

High-resolution mass spectra were recorded on a FTICR-MS Apex Ultra (BrukerDaltonics) 7 T instrument. NMR spectra were recorded on a Bruker 400 Ultra-Shield instrument (^{1}H 400 MHz, ^{13}C 100.61 MHz). ^{1}H and ^{13}C chemical shifts are expressed in δ (ppm), referenced to the solvent used, and the proton coupling constants *J* in hertz (Hz). Spectra were assigned using appropriate COSY, DEPT, HMQC, and HMBC sequences. Column chromatography was performed on silica gel (Merck 9385). Merck silica gel 60 F_{254} was used in analytical TLC, with visualization under UV light and by spraying either with Dragendorff's reagent or a solution of H_2SO_4–MeOH (1:1), followed by heating. Optical rotations were obtained using a Perkin Elmer 241 polarimeter, with quartz cells of 1 dm path length.

2.2. Plant material

The roots of *Tabernaemontana elegans* were collected in Mozambique, Maputo, during February, 2011. Taxonomical identification was performed by the Botanist Dr. Silva Mulhovo, Centro de Estudos Moçambicanos e de Etnociências, Universidade Pedagógica, Maputo, Mozambique. A voucher specimen (23/SM) has been deposited at the herbarium (LMA) of the Instituto de Investigação Agrária de Moçambique (IIAM), Maputo, Mozambique.

2.3. Extraction and isolation

The air dried powdered roots (3.5 kg) of *T. elegans* were extracted with MeOH as previously described (Mansoor et al., 2013). Briefly, the MeOH residue (500 g) was dissolved in Et_2O and extracted with 10% CH_3COOH. The pH of the acid layer was adjusted to 9 by the addition of dilute NH_4OH. The basic layer was successively extracted with CH_2Cl_2 and EtOAc, yielding the CH_2Cl_2 (90 g) and EtOAc (2 g) soluble fractions. The CH_2Cl_2 soluble fraction, the most cytotoxic, was subjected to silica gel column chromatography, using solvent mixtures of increasing polarity (*n*-hexane-EtOAc and EtOAc-MeOH), to yield fractions A–O. The crude fraction C (2.5 g) was chromatoghraphed on SiO_2 (*n*-hexane-EtOAc; 1:0 to 0:1), affording subfractions C_1-C_{20}. Fraction C_{16} (200 mg) was further fractionated by column chromatography (CH_2Cl_2/MeOH, from 1:0 to 9:1 v/v) giving six fractions (C_{16a}-C_{16f}). Fraction C_{16c} (35 mg) was purified by preparative TLC ($CHCl_3$/MeOH, 18:1), affording (16 mg) of compound **1**.

2.4. Cell culture

Human colon cancer cell lines, HCT116 and SW620 were grown in McCoy′s 5A medium and Dulbecco's modified Eagle's Medium (DMEM), respectively, both supplemented with 10% fetal bovine serum, and 1% antibiotic/antimycotic (Gibco, Life Technologies,

Paisley, UK) and maintained at 37ºC in a humidified atmosphere of 5% CO_2. HepG2 liver cancer cells were grown in DMEM supplemented with 10% fetal bovine serum, 1% nonessential amino acids, and 1% antibiotic/antimycotic (Invitrogen). CCD18co human colon fibroblasts (ATCC, CRL-1459) were grown in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum, 1% antibiotic/antimycotic, 2 mM GlutaMAX, 0.1 mM Non-essential amino acids (NEAA) (Invitrogen) and 0.57 mM Recombinant Human TNF-α (Peprotech, USA) and maintained at 37°C in a humidified atmosphere of 5% CO_2. Cells were seeded at a density of 5,000 cells/well in 96-well plates for MTS and caspase-3/7 activity assays; 50,000 cells/well in 24-well plates for Guava ViaCount assay; 150,000 cells/dish in 35 mm dishes for morphological evaluation of apoptosis; 1,500,000 cell/dish in 100 mm dishes for total protein extraction and immunoblot analysis.

2.5. Test compounds

Stock solutions of 1-50 µg/mL of crude methanolic extract, dichloromethane and ethyl acetate soluble alkaloid fractions (1-100 µg/mL), 0.5–50 mM of test compound (**1**), and positive control 5-FU (Sigma Chemical Co., St. Louis, MO, USA) were prepared in sterile dimethyl sulfoxide (DMSO; Sigma Chemical Co.). Twenty-four hours after cell plating, media was removed and replaced with fresh media containing samples and 5-FU at the indicated final concentrations, or DMSO vehicle control, for the indicated exposure times. The purity of **1** was at least 95% based on HPLC analysis and NMR spectroscopy.

2.6. MTS assay

Cell viability was evaluated by the CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA), using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS), according to the

manufacturer's instructions. The CellTiter96® Aqueous Assay is widely used to screen cancer cell sensitivity to commercially available, chemical synthesized or extracted test compounds (Borralho et al., 2009; Galluzzi, L, 2009; Silva et al., 2012). This assay is composed of solutions of MTS and an electron coupling reagent, phenazine methosulfate (PMS). MTS is bioreduced into a formazan product by dehydrogenase enzymes found in metabolic active cells. The amount of water-soluble formazan product can be measured by the amount of 490 nm absorbance, correlating with the number of viable cells in culture. For this purpose, after 72 h of cell treatment, 20 µL of MTS/PMS solution (19:1) was added to the culture medium and changes in absorbance were assessed using a GloMax-Multi+ Detection System (Promega). IC_{50} and 95% CI (Confidence Interval) values were calculated using GraphPad Prism v.5.00 (GraphPad Software, San Diego, CA, USA), where IC_{50} is the half maximal inhibitory concentration for a particular compound, and 95% CI represents a probability of 95% that the confidence interval produced will contain the true parameter value (IC_{50}).

2.7. Lactate dehydrogenase (LDH) assay

General cell death was measured by the Lactate Dehydrogenase Cytotoxicity Detection Kit^{PLUS} (Roche Applied Science, USA). In brief, cell culture supernatants were centrifuged at 250 × g for 2 min, and 50 µl of clear supernatant was transferred to a new 96 flat-bottom 96 well-plate. Cells attached in the well were lysed by adding 50 μ L of lysis solution diluted in culture medium to obtain a cell lysate. Subsequently, 50 µL of LDH reagent mix was added to each well containing either cell culture supernatant or cell lysate, followed by a 15 min incubation at room temperature, protected from light. Absorbance was read at 490 nm, with 620 nm reference wavelengths using a Model 680 microplate reader (Bio-Rad). The percentage of LDH release was determined as the ratio between released LDH (supernatant)

and the total LDH (supernatant + cell lysate), in the same well, as previously described (Tanaka et al., 2005).

2.8. Guava ViaCount assay

To evaluate viable, apoptotic, and dead cell populations in HCT116, SW620 and HepG2 cells exposed to compound **1** and vehicle controls, viaCount assay was used with the Guava easyCyte 5HT flow cytometer (GuavaTechnologies, Inc., Hayward, CA, USA). The ViaCount Assay distinguishes viable and non-viable cells based on differential permeability of two dyes in the Guava ViaCounts Reagent. The membrane-permeant dye that stains all nucleated cells, leaving the cellular debris unstained, while the membrane-impermeant dye brightly stains damaged cells, thus indicating apoptotic and dying cells. After 72h of treatment, cell culture supernatants were collected and adherent cells were detached with TrypLE (Invitrogen). Next, detached cells were pooled with cell culture supernatants and centrifuged for 5 min (650 *g*). Supernatants were discarded and the cells were resuspended in phosphate buffered saline (PBS). Subsequently, 15 µl of cell suspension were mixed with 135 µl of Guava ViaCount reagent, and incubated for 5 min at room temperature. Sample acquisition and data analysis were performed using the ViaCount software module.

2.9. Hoechst staining

Apoptotic nuclei were detected using DNA-binding stain Hoechst. In brief, cells were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS), pH 7.4, for 10 min at 25 ºC, and stained with 5 µg/mL Hoechst dye 33258 (Sigma) in PBS for 5 min. Cells were then washed with PBS and mounted using PBS:glycerol (3:1, v/v). Nuclear morphology was evaluated by fluorescence microscopy using an AxioScope.A1 microscope (Carl Zeiss Microscopy GmbH, Jena, Germany), under 400x magnification. A minimum of three random

microscopic fields with approximately 100 nuclei was counted for each condition. Fluorescent nuclei were scored and categorized according to the condensation and staining characteristics of chromatin and results were expressed as the percentage of apoptotic nuclei per field.

2.10. Caspase 3/7 activity assay

Caspase-3 and -7 activation status was measured using the Caspase-Glo 3/7 Assay (Promega). This assay is based on the cleavage of a luminogenic caspase-3/7 substrate, which contains the specific DEVD sequence. The presence of caspase-3 or -7 cleaves the luminogenic substrate, which in turn leads to release of the substrate for luciferase resulting in the production of a luminescent signal directly proportional to the amount of caspase activity present in the sample. For this purpose, 75 µL of Caspase-Glo 3/7 reagent was added to each well, and the mixture was incubated at room temperature for 30 min, leading to complete cell lysis, stabilization of substrate cleavage by caspases, and accumulation of luminescent signal. The resulting luminescence was measured using the GloMax-Multi+ Detection System (Promega).

2.11. Immunoblot analysis

After 72 h of compound exposure, cells were collected and processed for total protein extraction. Briefly, samples were homogenized in ice-cold 1:1 solution of buffer A (10 mM Tris–HCl, pH 7.6), 5 mM MgCl2, 1.5 mM KAc, 2 mM dithiothreitol (DTT) and Halt™ Protease and Phosphatase inhibitor cocktail, EDTA-free (#78445, Thermo Scientific) and buffer 2X (10 mM Tris–HCl pH 7.6, 1% Nonidet-P40 and Halt™ Protease and Phosphatase inhibitor cocktail), by vigorous vortexing and incubated on ice for 30 min. Next, samples were sonicated, and centrifuged at 10,000 g for 10 min, at 4°C. The clear supernatants containing the total protein extracts were transferred to a fresh tube and stored at -80°C.

Protein concentrations were determined using the Bio-Rad protein assay kit according to the manufacturer's instructions. Steady-state levels of XIAP, Bcl-2, and PARP proteins were determined by immunoblot analysis. Briefly, 50 µg of total protein extracts were separated on 10% SDS-polyacrylamide electrophoresis gels and transferred onto nitrocellulose membranes. After blocking with 5% milk solution, the blots were incubated overnight at 4ºC with primary mouse monoclonal antibody reactive to Bcl-2, (#sc-7382; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), or with rabbit polyclonal antibody reactive to XIAP and PARP (sc-11426 and sc-7150, respectively; Santa Cruz Biotechnology). Finally, membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin secondary antibodies (1:5000 dilution; Bio-Rad Laboratories) for 3 h at room temperature. The membranes were then processed for protein detection using Super Signal substrate (Pierce, Rockford, IL, USA). β-actin (#A-5441, Sigma Chemical Co.) was used as a loading control. The relative intensities of protein bands were quantified using the Image Lab densitometric analysis program (version 4.1; Bio-Rad).

2.12. Statistical analysis

All data were expressed as mean ± standard error of mean (SEM) from at least three independent experiments. Statistical analysis was performed using Student's t-test. Values of $p < 0.05$ were considered significant.

3. Results and discussion

3.1 Cytotoxic activity of the crude methanol extract and resulting alkaloid fractions

The air-dried powdered roots of *T. elegans* were exhaustively extracted with methanol. The methanol residue was submitted to acid/base extraction leading to alkaloid fraction, which was further partitioned into CH_2Cl_2 and ethyl acetate fractions (Mansoor et al., 2013).

The crude methanol extract and the resulting alkaloid fractions (CH_2Cl_2 and EtOAc) were assessed for their potential cytotoxicity in HCT116 and SW620 colon, HEPG2 liver cell lines, using the MTS assay. As shown in Fig. 1, the methanol crude extract and both alkaloid fractions showed significant cytotoxic activity against the three cell lines. The dichloromethane soluble alkaloid fraction displayed the strongest cytotoxic effects at lower concentrations, after 72 h of cell treatment. It inhibited 54, 43 and 59% of cell growth in HCT116 and SW620 colon, HEPG2 liver cell lines, respectively, at concentration of 1 µg/mL. The crude methanol extract and the ethyl acetate soluble alkaloid fraction were effective at concentration higher than 5 µg/mL.

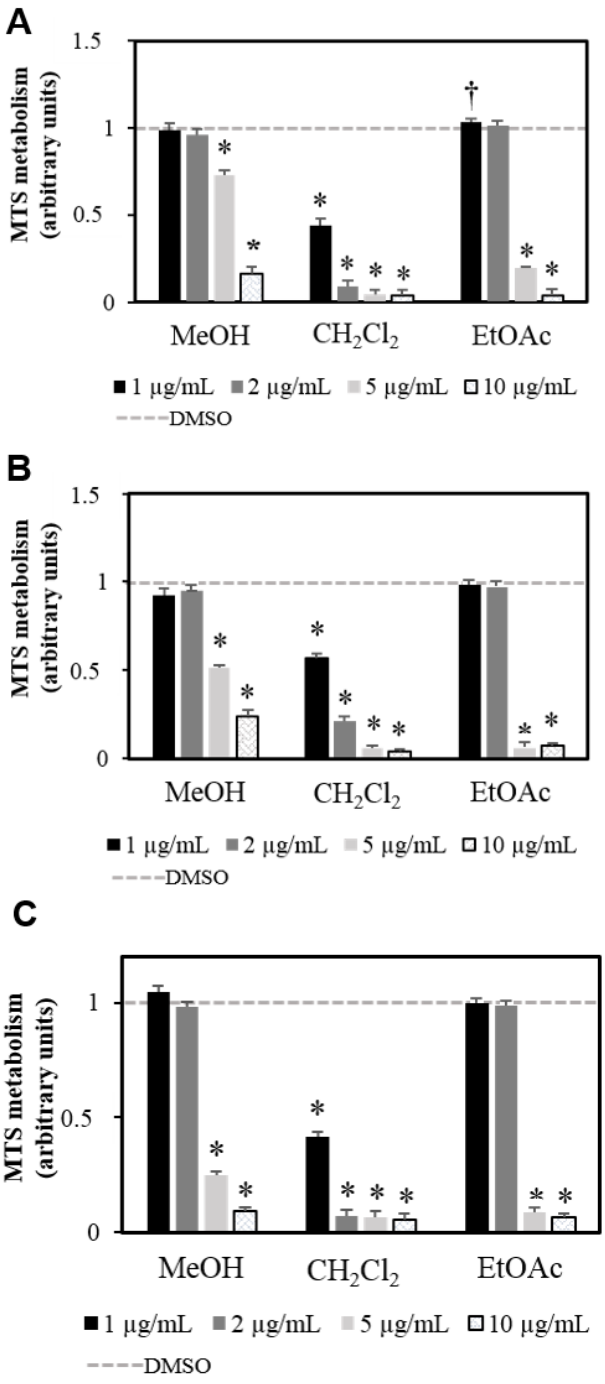
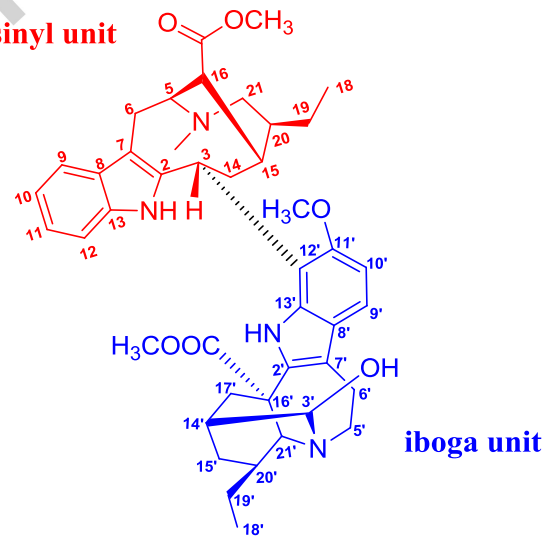


Fig. 1. Evaluation of extract effects on cell viability by the MTS assay. Cell viability was assessed by the MTS assay after exposing HCT116 (**A**) or SW620 (**B**) human colon cancer cells, and HepG2 liver cancer cells (**C**) to 1, 2, 5, 10 µg/mL of MeOH crude extract and CH_2Cl_2 and EtOAc soluble alkaloid fractions for 72 h, and DMSO vehicle control. Results are expressed as mean ± SEM fold change to vehicle control treated cells. *$p < 0.01$ and $^{\dagger}p < 0.05$ from vehicle control.

3.2. Identification of (3'R)-hydroxytaberanelegantine

Repeated fractionation of the dichloromethane soluble alkaloid fraction, the most cytotoxic, yielded the monoterpene indole alkaloids dregamine, tabernaemontanine, 16-epidregamine, voacangine, vobasine and the bisindoles tabernaelegantine C and tabernaelegantinine B (Mansoor et al., 2013). Further study of the referred fraction gave rise to compound **1** (Fig. 2). Compound **1** has shown a molecular formula of $C_{43}H_{54}N_4O_6$, by HRMS-ESI-TOF m/z 723.40985 $[M + H]^+$ (calcd. for $C_{43}H_{55}N_4O_6$, 723.41161), and $[\alpha]^{20}$ –4.6. The chemical structure of **1**, a vobasinyl-iboga bisindole alkaloid, was identified as (3'*R*)-hydroxytaberanelegantine C, based on its physical and spectroscopic data, including two-dimensional NMR experiments (COSY, HMQC, and HMBC; Supplementary Materials), and literature data (Girardot et al., 2012). This compound was recently isolated for the first time, from the stem bark of *Tabernaemontana sessifolia* (Baker) (Girardot et al., 2012). Dimeric indole alkaloids of the vobasinyl-iboga type exist in abundance in the genus *Tabernaemontana*. They are biosynthetized through the reaction of the vobasinyl cation, highly reactive, with a nucleophilic aromatic carbon of the ibogan moiety. Usually, the linkage occurs between C-3 of the vobasinyl unity and C-10', C-11' or C-12' of the ibogan moiety (Cordell, 2006).



1

Fig. 2. (3'*R*)-hydroxytaberanelegantine C (**1**) isolated from the roots of *T. elegans*.

3.3. Cytotoxic activity of (3'R)-hydroxytaberanelegantine C

To study the cytotoxic effect of (3'*R*)-hydroxytaberanelegantine C (**1**), HCT116 and SW620 colon, HEPG2 liver and CCD18co human colon fibroblasts cell lines were treated with increasing concentrations of compound **1** or 5-FU (a cornerstone agent in colon cancer treatment and a well-characterized apoptosis inducer) and the cytotoxic activity was initially determined by MTS metabolism assay, calculating the half maximal inhibitory concentration IC_{50}. After 72 h of treatment of cells the IC_{50} values were 3.20 µM and 2.49 µM, respectively, in HCT116 cells; 3.49 µM and 5.39 µM, respectively, in SW620 cells; 3 µM and 12.2 µM, respectively, in HepG2 cells; and 10.17 µM and > 100 µM, respectively, in CCD18co cells (Table 1). Therefore, some degree of selectivity (SI > 2.91; Table 1) was observed toward the cancer cell lines tested, compared to normal colon fibroblasts.

Subsequently, HCT116, SW620 and HepG2 cells were exposed to IC_{50} and 2-fold IC_{50} values of **1** or 5-FU, for 72 h, using as control cells exposed to vehicle (DMSO), and cells without exposure to compound nor vehicle (no addition). General cell death was assessed by the lactate dehydrogenase (LDH) assay. This assay measures the enzymatic activity of LDH release from damaged cells. Cell death is generally associated with damage of plasma membrane, leading to release of intracellular content into extracellular milieu. The presence of LDH in cell culture supernatant results from damaged cytoplasmic membrane (Goergen et al., 1993). The results obtained showed that compound **1** exposure at IC_{50} concentration resulted in 1.3- to 2-fold increased cell death in HCT116 and HepG2, respectively, compared to vehicle (DMSO) exposure ($p < 0.01$) (Fig. 3A and 3C). However, exposure of SW620 cells to **1** at IC_{50} did not lead to increased LDH release (Fig. 3B). In parallel, **1** exposure at 2-fold IC_{50} increased cell death by 3.5- to 4-fold compared to vehicle (DMSO) control ($p < 0.01$), and 2.5-fold compared to 5-FU at equitoxic concentration in the three cell lines (Fig. 3).

Table 1. IC_{50} values of **1** and positive control 5-FU as assessed by the MTS assay

	Compounds						
	1				**5-FU**		
Cell lines	**IC_{50} (μM)[a]**	**IC_{50} (μg/mL)[a]**	**95% CI**	**SI[b]**	**IC_{50} (μM)[a]**	**95% CI**	**SI[b]**
HCT116	3.20	2.31	3.60-3.69	3.18	2.49	9.28-11.1	>40
SW620	3.49	2.52	3.41-3.56	2.91	5.39	2.18-3.67	18.55
HepG2	3.00	2.17	2.86-2.95	3.39	12.2	10.6-14.1	8.20
CCD18co	10.17	7.35	9.28-11.16	-	>100	-	-

[a] After exposure of HCT116, SW620 and HepG2 cancer cells to **1**, 5-FU and DMSO vehicle control for 72 h, from at least three independent experiments, where 95% CI represents 95% confidence intervals.

[b] Selectivity index (SI) = IC_{50} CCD18co / IC_{50} cancer cells.

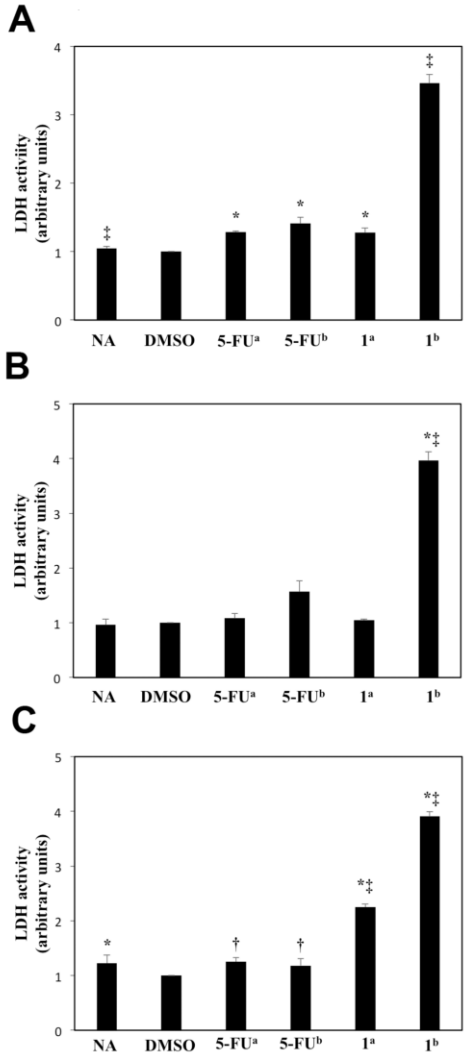


Fig. 3. General cell death as assessed by the LDH assay after exposure of HCT116 (**A**) or SW620 (**B**) human colon cancer cells, or HepG2 liver cancer cells (**C**) to **1**, 5-FU, or DMSO vehicle control for 72 h. Results are expressed as mean ± SEM of at least three different experiments. [a]IC_{50} concentrations, [b]2-fold IC_{50} concentrations. $^{*}p < 0.01$ and $^{\dagger}p < 0.05$ from vehicle control, $^{\ddagger}p < 0.01$ from 5-FU.

3.4. Apoptosis induction activity of (3'R)-hydroxytaberanelegantine C

3.4.1. Apoptosis assessment by Guava ViaCount assay

Induction of apoptosis in cancer cells has been considered crucial in anticancer therapy. Therefore, flow cytometry analysis was performed, using the Guava ViaCount assay, to detect viable, mid apoptotic and dead cells following exposure to **1** and 5-FU. The results demonstrated that **1** significantly induced cell death following 72 h exposure to IC_{50} and 2-fold IC_{50} concentrations, in all cancer cell lines ($p < 0.01$) (Fig. 4). In particular, **1** at IC_{50} concentration was able to significantly increase the incidence of apoptosis in HCT116, SW620 and HepG2 cells up to 20%, as compared to vehicle control exposure ($p < 0.01$). Further, at 2-fold IC_{50} concentration, compound **1** increased apoptotic cells up to 40% in HCT116 and SW620 cells, and up to 30% in HepG2 cells ($p < 0.01$) (Fig. 4). Importantly, 5-FU exposure at IC_{50} or 2-fold IC_{50} concentrations induced significantly less apoptotic cell death as compared to **1** ($p < 0.01$). Therefore, these results demonstrate a higher apoptosis induction by **1** compared to 5-FU, in HCT116, SW620 and HepG2 cancer cell lines.

3.4.2 Nuclear morphological evaluation

Subsequently, nuclear morphological evaluation studies were carried out by fluorescent microscopy following Hoechst staining to validate apoptosis induction by **1**. Normal nuclei showed non-condensed chromatin dispersed over the entire nucleus. Apoptotic nuclei were identified by condensed chromatin, contiguous to the nuclear membrane, as well as nuclear fragmentation of condensed chromatin. In fact, compound **1** treatment induced higher levels of apoptosis in the cell lines tested. Cell exposure to IC_{50} concentration of **1** for 24 h, led to 6, 9 and 25% apoptotic cells, respectively in HCT116, SW620 and HepG2 cells, compared to vehicle-treated control cells, which displayed less than 3% of apoptotic cells ($p < 0.01$). Further, **1** exposure at 2-fold IC_{50} concentration led to 35, 20 and 25% apoptotic cells, in

HCT116, SW620 and HepG2 cells, respectively, as compared to controls. Apoptosis elicited by **1** was significantly higher than that induced by the known apoptosis-inducing agent 5-FU, whose exposure at IC_{50} and 2-fold IC_{50} led to less than 7% apoptosis in all the cell lines (Fig. 5).

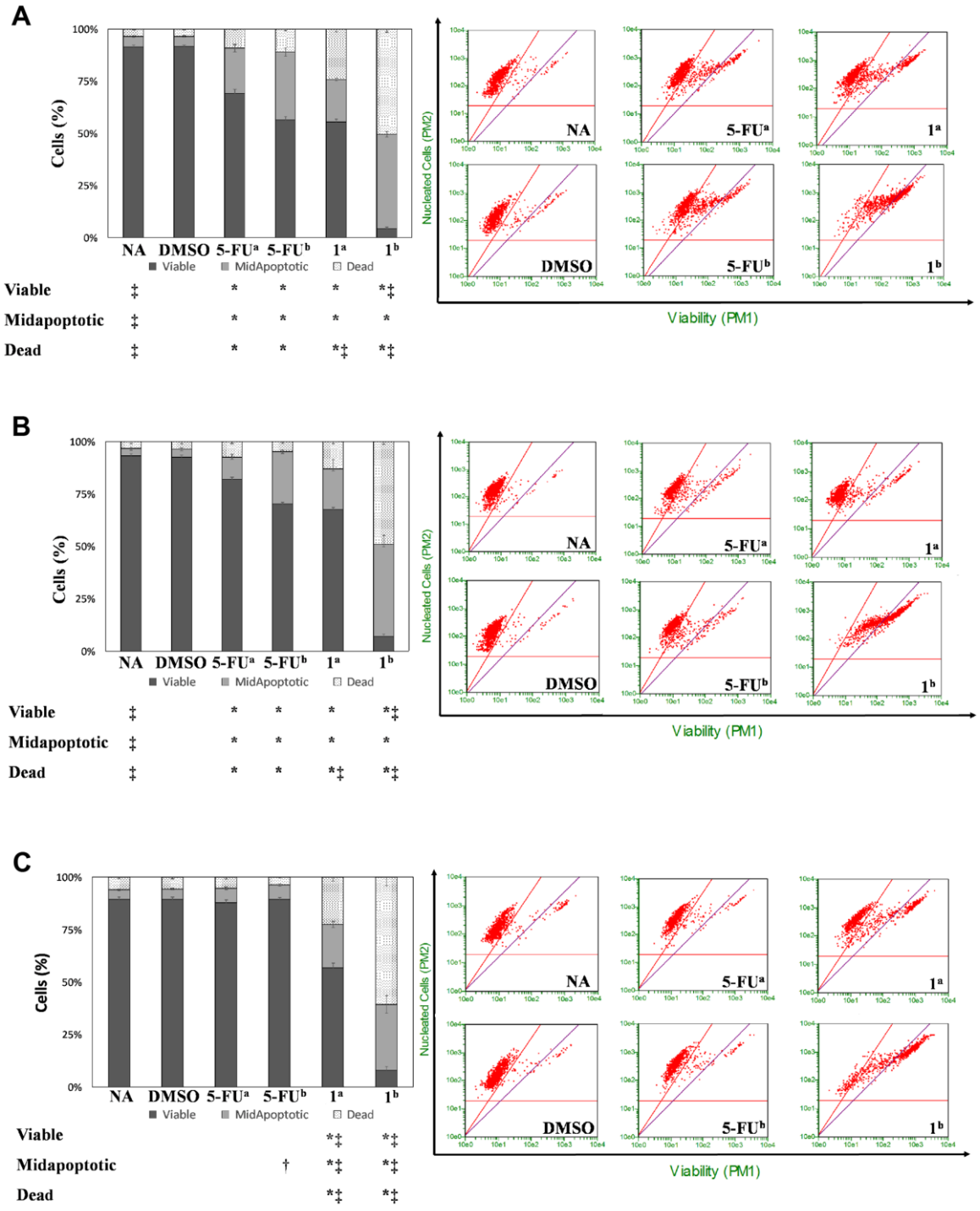


Fig. 4. Evaluation of compound effect on cell viability using the Guava ViaCount assay. Cell populations obtained by Guava ViaCount flow cytometry after 72 h incubation of HCT116 (**A**) or SW620 (**B**) human colon cancer cells, and HepG2 human liver cancer cells (**C**) with **1**, 5-FU, or DMSO vehicle control. Results are expressed as percentage (%) of viable, mid-apoptotic, or dead cells ± SEM of at least three different experiments. [a]IC_{50} concentration, [b]2-fold IC_{50} concentration. $^{*}p < 0.01$ and $^{\dagger}p < 0.05$ from vehicle control; $^{\ddagger}p < 0.01$ from 5-FU.

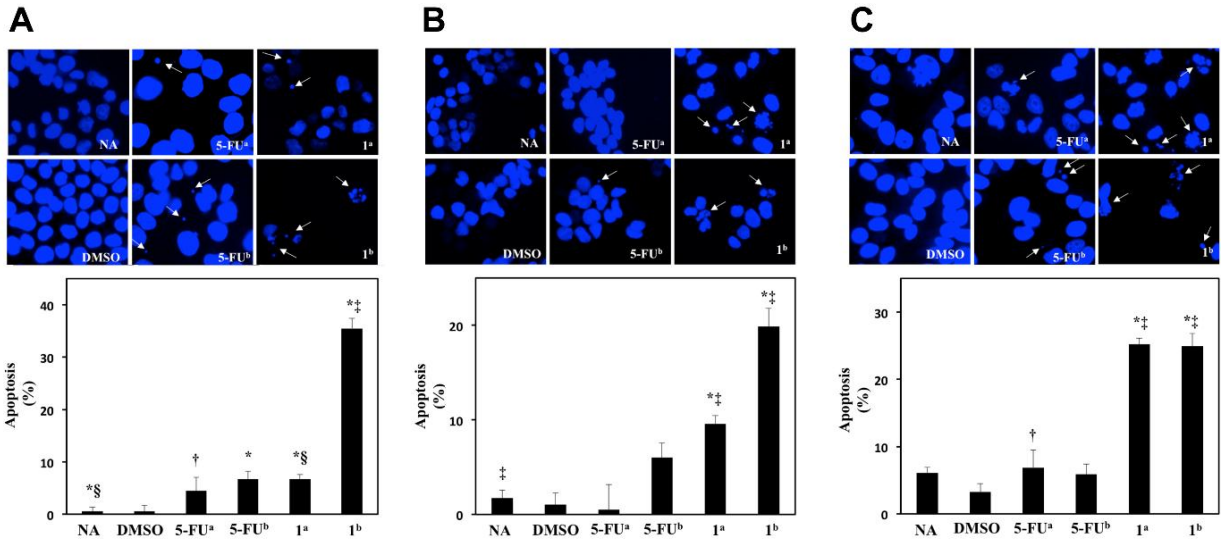


Fig. 5. Evaluation of compound **1** effect on apoptosis. Evaluation of changes in nuclear morphology by fluorescence microscopy of Hoechst stained nuclei, after 24 h incubation of HCT116 (**A**) or SW620 (**B**) human colon cancer cells, and HepG2 liver cancer cells (**C**) with **1**, 5-FU, or DMSO vehicle control. Representative images of compound effect on apoptosis, with white arrows highlighting apoptotic cells (upper panels; 400x magnification). Quantification of apoptosis induced by compound exposure (lower panels). Results are expressed as mean ± SEM of at least three different experiments. [a]IC_{50} concentration, [b]2-fold IC_{50} concentration. $^{*}p < 0.01$ and $^{\dagger}p < 0.05$ from vehicle control, $^{\ddagger}p < 0.01$ and $^{\S}p < 0.05$ from 5-FU.

3.4.3. Caspase 3/7 activity evaluation

Apoptosis induction by compound **1** was further confirmed by caspase-3/7 activity assays, which corroborated the nuclear morphology evaluation data. Indeed compound **1** at IC_{50} concentration increased caspase-3/7 activity by 1.3-fold, compared with DMSO vehicle control and 5-FU, in the three cell lines (Fig. 6). Further, in HCT116 cells, compound **1** at 2-fold IC_{50} increased caspase-3/7 activity by 2.5- and 2-fold, respectively, compared with DMSO vehicle control and 5-FU treated cells (Fig. 6A). Compound **1** also increased caspase-3/7 activity by 1.3-fold in SW620 and HepG2, compared both with the DMSO vehicle control and 5-FU (Fig. 6B and 6C).

3.4.4. Immunoblot analysis of apoptosis-related proteins

To explore the molecular pathways by which **1** exerts its pro-apoptotic effect, key apoptosis proteins were evaluated by immunoblot analysis, using total protein extracts from HCT116, SW620 or HepG2 cells exposed to **1** or 5-FU at IC_{50} or 2-fold IC_{50} for 72 h, and vehicle control (Fig. 7). The data obtained showed that exposure to **1** in all three cancer cell lines led to increased cleavage of the endogenous substrate of active caspase-3, poly(ADP-ribose) polymerase (PARP). Further, steady-state levels of anti-apoptotic proteins XIAP and Bcl-2, were reduced (Fig. 7). In contrast, p53 protein expression was not increased (data not shown). These data suggest that survival signaling is somehow inhibited thus contributing to compound 1-induced apoptosis. DNA damage, in turn, does not appear to play a role in apoptosis induction.

Therefore, increased PARP processing after **1** exposure confirmed data from Guava ViaCount and Hoechst staining, while being associated with reduced expression of anti-apoptotic proteins.

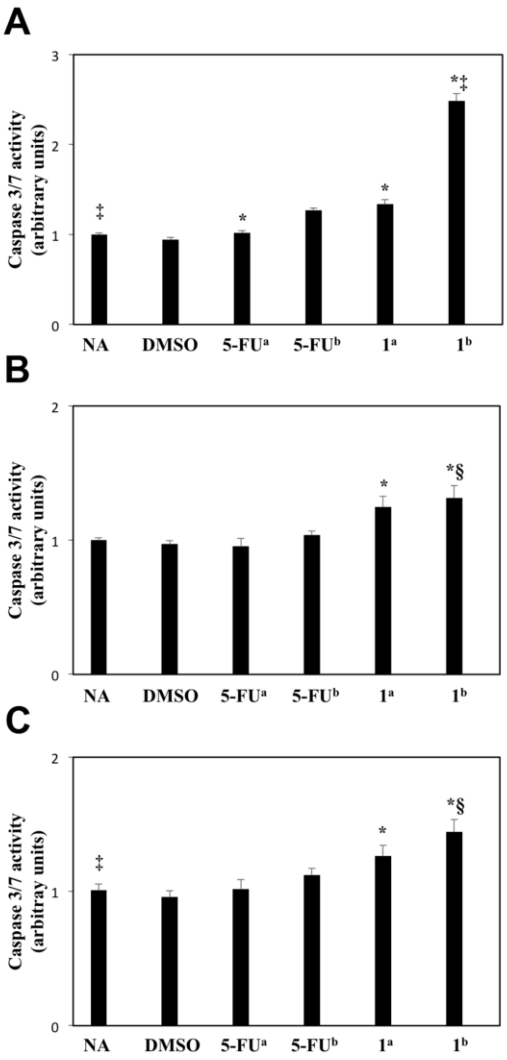


Fig. 6. Evaluation of compound effect on caspase-3/7 activity. Caspase-3/7 activity after exposure of HCT116 (**A**) or SW620 (**B**) human colon cancer cells, and HepG2 liver cancer cells (**C**) to **1** and 5-FU or DMSO vehicle control, for 24 h. Results are expressed as mean ± SEM of at least three different experiments. [a]IC_{50} concentration, [b]2-fold IC_{50} concentration. [*]$p < 0.01$ and [†]$p < 0.05$ from vehicle control, [‡]$p < 0.01$ and [§]$p < 0.05$ from 5-FU.

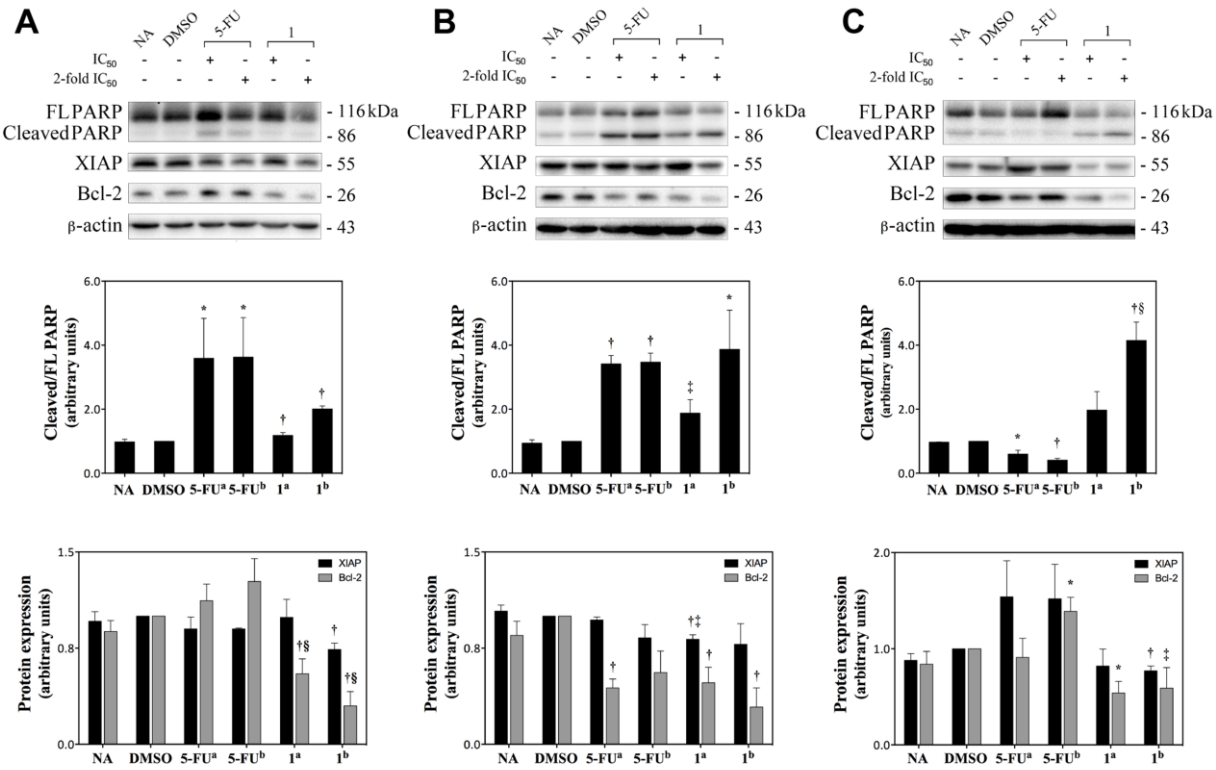


Fig. 7. Effects of **1** or 5-FU exposure on steady-state expression of cell death associated proteins. HCT116 (**A**) or SW620 (**B**) human colon cancer cells, and HepG2 liver cancer cells (**C**) were exposed to **1**, 5-FU, or DMSO vehicle control for 72 h. Total proteins extracts were separated in 10% SDS-PAGE gels and submitted to immunoblot analysis. Representative blots for PARP cleavage and steady-state protein levels of XIAP and Bcl-2 (top), and corresponding histograms (bottom). Results are expressed as mean ± SEM from at least three independent experiments. [a]IC_{50} concentration, [b]2-fold IC_{50} concentration. $^{*}p < 0.01$ and $^{\dagger}p < 0.05$ from vehicle control, $^{\ddagger}p < 0.01$ and $^{\S}p < 0.05$ from 5-FU.

4. Conclusions

To summarize, in this study compound **1** showed remarkable induction of apoptosis in HCT116, SW620 and HepG2 cells as compared to the cornerstone agent in colon cancer treatment, 5-FU, thus contributing for the validation of *T. elegans* as a medicinal plant for

cancer treatment. The apoptosis inducing profile of **1** not only in HCT116 colon cancer cells, but also in SW620 colon and HepG2 liver carcinoma cells, which are less sensitive to 5-FU, highlights its potential as a promising lead for MDR-reversing anticancer drug development.

Acknowledgements

This study was financially supported by Fundação para a Ciência e a Tecnologia (FCT), Portugal (projects: PTDC/QEQ-MED/0905/2012; PhD grant SFRH/BD/77612/2011). The authors thank the Portuguese Embassy in Mozambique, as well as the Portuguese Office of International Affairs for plant transport. We also acknowledge Prof. Carlos Cordeiro, Faculdade de Ciências, Universidade de Lisboa, for high resolution mass data (FCT, REDE/1501/REM/2005).

Conflict of Interest

The authors declare that there are no conflicts of interest.

Ethics

There is no ethical issue with this study.

Supplementary Materials

Supplementary material associated with this article can be found, in the online version.

References

Baird, R.D., Kaye, S.B., 2003. Drug resistance reversal-are we getting closer? Eur. J. Cancer 39, 2450–2461.

Bhanot, A., Sharma, R., Noolvi, M.N., 2011. Natural sources as potential anti-cancer agents: A review. Int. J. Phytomedicine 3, 9-26.

Borralho, P.M., Kren, B.T., Castro, R.E., da Silva, I.B., Steer, C.J., Rodrigues, C.M.P., 2009. MicroRNA-143 reduces viability and increases sensitivity to 5-fluorouracil in HCT116 human colorectal cancer cells. FEBS J. 276, 6689-6700.

Carbone, A., Parrino, B., Di Vita, G., Attanzio, A., Spanò, V., Montalbano, A., Barraja, P., Tesoriere, L., Livrea, M.A., Diana, P., Cirrincione, G., 2015. Synthesis and Antiproliferative Activity of Thiazolyl-bis-pyrrolo[2,3-b]pyridines and Indolyl-thiazolyl-pyrrolo[2,3-c]pyridines, Nortopsentin Analogues. Mar. Drugs 13, 460-492 .

Carbone, A., Pennati, M., Parrino, B., Lopergolo, A., Barraja, P., Montalbano, A., Spanò, V., Sbarra, S., Doldi, V., De Cesare, M., Cirrincione, G., Diana, P., Zaffaroni, N., 2013. Novel 1H-Pyrrolo[2,3-b]pyridine Derivative Nortopsentin Analogues: Synthesis and Antitumor Activity in Peritoneal Mesothelioma Experimental Models. J. Med. Chem. 56, 7060–7072.

Cordell, G.A., 2006. The alkaloids: chemistry and biology. Academic Press. (Vol.63) pp. 258-270.

Chhabra, S.C., Mahunnah, B.L.A., Mshiu, E.N., 1987. Plants used in traditional medicine in Eastern Tanzania. I. Pteridophytes and Angiosperms (Acanthaceae to Canellaceae). J. Ethnopharmacol. 21, 253–277.

Cragg, G.M., Grothaus, P.G., Newman, D.J., 2009. Impact of natural products on developing new anti-cancer agents. Chem. Rev. 109, 3012-3043.

Cragg, G.M., Newman, D.J., 2005. Plants as a source of anti-cancer agents. J. Ethnopharmacol. 100, 72-79.

de Sa, A., Fernando, R., Barreiro, E.J., Fraga, M., Alberto, C., 2009. From nature to drug discovery: The indole scaffold as a “privileged structure.” Mini Rev. Med. Chem. 9, 782-793.

Duarte, N., Varga, A., Cherepnev, G., Radics, R., Molnár, J., Ferreira, M.J.U., 2007. Apoptosis induction and modulation of P-glycoprotein mediated multidrug resistance by new macrocyclic lathyrane-type diterpenoids. Bioorg. Med. Chem. 15, 546-554.

Elmore, S., 2007. Apoptosis: A review of programmed cell death. Toxicol. Pathol. 35, 495-516.

Ferlay, J., Soerjomataram, I., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D.M., Forman, D., Bray, F., 2015. Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. Int. J. Cancer 136, E359-E386.

Ferreira, M.J.U., Duarte, N., Reis, M., Madureira, A.M., Molnár, J., 2014. *Euphorbia* and *Momordica* metabolites for overcoming multidrug resistance. Phytochem. Rev. 13, 915-935.

Fulda, S., 2015. Targeting extrinsic apoptosis in cancer: Challenges and opportunities. Semin. Cell Dev. Biol. 39, 20-25.

Galluzzi, L.; Aaronson, S. A.; Abrams, J.; Alnemri, E. S.; Andrews, D. W.; Baehrecke, E. H.; Bazan, N. G.; Blagosklonny, M. V.; Blomgren, K.; Borner, C.; Bredesen, D. E.; Brenner, C.; Castedo, M.; Cidlowski, J. A.; Ciechanover, A.; Cohen, G. M.; De Laurenzi, V.; De Maria, R.; Deshmukh, M.; Dynlacht, B. D.; El-Deiry, W. S.; Flavell, R. A.; Fulda, S.; Garrido, C.; Golstein, P.; Gougeon, M. L.; Green, D. R.; Gronemeyer, H.; Hajnóczky, G.; Hardwick, J. M.; Hengartner, M. O.; Ichijo, H.; Jäättelä, M.; Kepp, O.; Kimchi, A.; Klionsky, D. J.; Knight, R. A.; Kornbluth, S.; Kumar, S.; Levine, B.; Lipton, S. A.; Lugli, E.; Madeo, F.; Malorni, W.; Marine, J. C. W.; Martin, S. J.; Medema, J. P.; Mehlen, P.; Melino, G.; Moll, U. M.; Morselli, E.; Nagata, S.; Nicholson, D. W.; Nicotera, P.; Nuñez, G.; Oren. M.; Penninger, J.; Pervaiz, S.; Peter, M. E.; Piacentini, M.; Prehn, J. H. M.; Puthalakath, H.; Rabinovich, G. A.; Rizzuto, R.; Rodrigues, C. M. P.; Rubinsztein, D. C.; Rudel, T.; Scorrano, L.; Simon, H. U.; Steller, H.; Tschopp, J.; Tsujimoto, Y.; Vandenabeele, P.; Vitale, I.; Vousden, K. H.; Youle, R. J.; Yuan, J.; Zhivotovsky, B.; Kroemer, G., 2009. Guidelines for the use and

interpretation of assays for monitoring cell death in higher eukaryotes. Cell Death Differ. 16, 1093–1107.

Girardot, M., Gadea, A., Deregnaucourt, C., Deville, A., Dubost, L., Nay, B., Maciuk, A., Rasoanaivo, P., Mambu, L., 2012. Tabernaelegantinals: Unprecedented cytotoxic bisindole alkaloids from *Muntafara sessilifolia*. Eur. J. Org. Chem. 2012, 2816-2823.

Goergen, J.L., Marc, A., Engasser, J.M., 1993. Determination of cell lysis and death kinetics in continuous hybridoma cultures from the measurement of lactate dehydrogenase release. Cytotechnology 11, 189-195.

Gottesman, M.M., Fojo, T., Bates, S.E., 2002. Multidrug resistance in cancer: role of ATP-dependent transporters. Nat. Rev. Cancer 2, 48-58.

Ishikura, M., Abe, T., Choshib, T., Hibinob, S., 2013. Simple indole alkaloids and those with a non-rearranged monoterpenoid unit. Nat. Prod. Rep. 30, 694-752.

Lage, H., 2008. An overview of cancer multidrug resistance: A still unsolved problem. Cell. Mol. Life Sci. 65, 3145-3167.

Leonard, J., 1999. Recent progress in the chemistry of monoterpenoid indole alkaloids derived from secologanin. Nat. Prod. Rep. 16, 319-338.

Mansoor, T.A., Borralho, P.M., Dewanjee, S., Mulhovo, S., Rodrigues, C.M.P., Ferreira, M.-J.U., 2013. Monoterpene bisindole alkaloids, from the African medicinal plant *Tabernaemontana elegans*, induce apoptosis in HCT116 human colon carcinoma cells. J. Ethnopharmacol. 149, 463-470.

Mansoor, T.A., Ramalho, R.M., Luo, X., Ramalhete, C., Rodrigues, C.M.P., Ferreira, M.J.U., 2011. Isoflavones as apoptosis inducers in human hepatoma HuH-7 cells. Phytother. Res. 25, 1819-1824.

Mansoor, T.A., Ramalho, R.M., Rodrigues, C.M.P., Ferreira, M.-J.U., 2012. Dibenzylbutane- and butyrolactone-type lignans as apoptosis inducers in human hepatoma HuH-7 cells. Phytother. Res. 26, 692-696.

Matos, A.M., Reis, M., Duarte, N., Spengler, G., Molnár, J., Ferreira, M.J.U., 2015. Epoxylathyrol derivatives: Modulation of ABCB1-mediated multidrug resistance in human colon adenocarcinoma and mouse T-lymphoma cells. J. Nat. Prod. 78, 2215-2228.

Mishra, B.B., Tiwari, V.K., 2011. Natural products: An evolving role in future drug discovery. Eur. J. Med. Chem. 46, 4769-4807.

Newman, D.J., Cragg, G.M., 2012. Natural products as sources of new drugs over the 30 years from 1981 to 2010. J. Nat. Prod. 75, 311-335.

Paterna, A., Borralho, P.M., Gomes, S.E., Mulhovo, S., Rodrigues, C.M.P., Ferreira, M.J.U., 2015. Monoterpene indole alkaloid hydrazone derivatives with apoptosis inducing activity in human HCT116 colon and HepG2 liver carcinoma cells. Bioorg. Med. Chem. Lett. 25, 3556-3559.

Reis, M., Ferreira, R.J., Santos, M.M.M., dos Santos, D.J.V.A., Molnár, J., Ferreira, M.J.U., 2013. Enhancing macrocyclic diterpenes as multidrug-resistance reversers: structure-activity studies on jolkinol D derivatives. J. Med. Chem. 56, 748-760.

Shmelzer, G.H., Gurib-Fakim, A. 2008. Plant Resource of Tropical Africa 11 (1). Medicinal Plants 1. PROTA Fundation, Backhuys Publishers, CTA, Wageningen, Netherlands.

Silva, T.F.S., Martins, L.M.D.R.S., da Silva, M.F.C., Fernandes, A.R., Silva, A., Borralho, P.M., Santos, S., Rodrigues, C.M.P., Pombeiro, A.J.L., 2012. Cobalt complexes bearing scorpionate ligands: synthesis, characterization, cytotoxicity and DNA cleavage. Dalt. Trans. 41, 12888-12897.

Tanaka, M., Arai, H., Liu, N., Nogaki, F., Nomura, K., Kasuno, K., Oida, E., Kita, T., Ono, T., 2005. Role of coagulation factor Xa and protease-activated receptor 2 in human mesangial cell proliferation. Kidney Int. 67, 2123-2133.

Vanbeek, T.A., Verpoorte, R., Svendsen, A.B., Leeuwenberg, A.J.M., Bisset, N.G., 1984. Pharmacognostical studies on *Tabernaemontans* species. 8. *Tabernaemontana L.*

(Apocynaceae): A review of its taxonomy, phytochemistry, ethnobotany and pharmacology. J. Ethnopharmacol. 10, 1-156.

Fig. 1. Evaluation of extract effects on cell viability by the MTS assay. Cell viability was assessed by the MTS assay after exposing HCT116 (**A**) or SW620 (**B**) human colon cancer cells, and HepG2 liver cancer cells (**C**) to 1, 2, 5, 10 μg/mL of MeOH crude extract and CH_2Cl_2 and EtOAc soluble alkaloid fractions for 72 h, and DMSO vehicle control. Results are expressed as mean ± SEM fold change to vehicle control treated cells. $^{*}p < 0.01$ and $^{\dagger}p < 0.05$ from vehicle control.

Fig. 2. (3'*R*)-hydroxytaberanelegantine C (**1**) isolated from the roots of *T. elegans*.

Fig. 3. General cell death as assessed by the LDH assay after exposure of HCT116 (**A**) or SW620 (**B**) human colon cancer cells, or HepG2 liver cancer cells (**C**) to **1**, 5-FU, or DMSO vehicle control for 72 h. Results are expressed as mean ± SEM of at least three different experiments. [a]IC_{50} concentrations, [b]2-fold IC_{50} concentrations. $^{*}p < 0.01$ and $^{\dagger}p < 0.05$ from vehicle control, $^{\ddagger}p < 0.01$ from 5-FU.

Fig. 4. Evaluation of compound effect on cell viability using the Guava ViaCount assay. Cell populations obtained by Guava ViaCount flow cytometry after 72 h incubation of HCT116 (**A**) or SW620 (**B**) human colon cancer cells, and HepG2 human liver cancer cells (**C**) with **1**, 5-FU, or DMSO vehicle control. Results are expressed as percentage (%) of viable, mid-

apoptotic, or dead cells ± SEM of at least three different experiments. [a]IC_{50} concentration, [b]2-fold IC_{50} concentration. $^{*}p < 0.01$ and $^{\dagger}p < 0.05$ from vehicle control; $^{\ddagger}p < 0.01$ from 5-FU.

Fig. 5. Evaluation of compound **1** effect on apoptosis. Evaluation of changes in nuclear morphology by fluorescence microscopy of Hoechst stained nuclei, after 24 h incubation of HCT116 (**A**) or SW620 (**B**) human colon cancer cells, and HepG2 liver cancer cells (**C**) with **1**, 5-FU, or DMSO vehicle control. Representative images of compound effect on apoptosis, with white arrows highlighting apoptotic cells (upper panels; 400x magnification). Quantification of apoptosis induced by compound exposure (lower panels). Results are expressed as mean ± SEM of at least three different experiments. [a]IC_{50} concentration, [b]2-fold IC_{50} concentration. $^{*}p < 0.01$ and $^{\dagger}p < 0.05$ from vehicle control, $^{\ddagger}p < 0.01$ and $^{\S}p < 0.05$ from 5-FU.

Fig. 6. Evaluation of compound effect on caspase-3/7 activity. Caspase-3/7 activity after exposure of HCT116 (**A**) or SW620 (**B**) human colon cancer cells, and HepG2 liver cancer cells (**C**) to **1** and 5-FU or DMSO vehicle control, for 24 h. Results are expressed as mean ± SEM of at least three different experiments. [a]IC_{50} concentration, [b]2-fold IC_{50} concentration. $^{*}p < 0.01$ and $^{\dagger}p < 0.05$ from vehicle control, $^{\ddagger}p < 0.01$ and $^{\S}p < 0.05$ from 5-FU.

Fig. 7. Effects of **1** or 5-FU exposure on steady-state expression of cell death associated proteins. HCT116 (**A**) or SW620 (**B**) human colon cancer cells, and HepG2 liver cancer cells (**C**) were exposed to **1**, 5-FU, or DMSO vehicle control for 72 h. Total proteins extracts were separated in 10% SDS-PAGE gels and submitted to immunoblot analysis. Representative blots for PARP cleavage and steady-state protein levels of XIAP and Bcl-2 (top), and corresponding histograms (bottom). Results are expressed as mean ± SEM from at least three

independent experiments. [a]IC_{50} concentration, [b]2-fold IC_{50} concentration. [*]$p < 0.01$ and [†]$p < 0.05$ from vehicle control, [‡]$p < 0.01$ and [§]$p < 0.05$ from 5-FU.

Table 1. IC_{50} values of **1** and positive control 5-FU as assessed by the MTS assay

	Compounds						
	1				**5-FU**		
Cell lines	**IC_{50} (μM)[a]**	**IC_{50} (μg/mL)[a]**	**95% CI**	**SI[b]**	**IC_{50} (μM)[a]**	**95% CI**	**SI[b]**
HCT116	3.20	2.31	3.60-3.69	3.18	2.49	9.28-11.1	>40
SW620	3.49	2.52	3.41-3.56	2.91	5.39	2.18-3.67	18.55
HepG2	3.00	2.17	2.86-2.95	3.39	12.2	10.6-14.1	8.20
CCD18co	10.17	7.35	9.28-11.16	-	>100	-	-

[a] After exposure of HCT116, SW620 and HepG2 cancer cells to **1**, 5-FU and DMSO vehicle control for 72 h, from at least three independent experiments, where 95% CI represents 95% confidence intervals.

[b] Selectivity index (SI) = IC_{50} CCD18co / IC_{50} cancer cells.

Graphical Abstract