Novel Cytotoxic Isolated from Jamaican *Hyptis verticillata* jacq Induces Apoptosis and Overcomes Multidrug Resistance

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Abstract. *Background: Adult T-cell leukemia is an aggressive hematological malignancy with a poor clinical prognosis, and a rapid resistance to chemotherapy is rapid. Materials and Methods: Cytotoxicity assay-directed fractionation identified a novel lignan-related agent, 4-methoxy-9-(3,4,5-trimethoxyphenyl)-8,9-dihydrofuro[3',4':6,7]naphtho[2,3-d][1,3]dioxol-6(5H)-one (4-MTDND) from the Jamaican plant Hyptis verticillata jacq, and its effects on apoptosis, cell cycle and drug resistance were elucidated. Results: The novel agent, 4-MTDND, exhibited cytotoxicity against myriad cancer types, with a wide therapeutic index of 30- to 40-fold, promoted G_2/M arrest and up-regulated expression of pro-apoptotic proteins p53 and BAX, as well as enhanced activation of caspase-3, caspase-9 and poly (ADP ribose) polymerase, consistent with apoptosis induction. Multidrug-resistant cancer cells were as susceptible to 4-MTDND as their non-resistant control counterparts, with 4-MTDND having greater efficacy compared to standard chemotherapy agents etoposide and mitoxantrone. Conclusion: The novel cytotoxic agent 4-MTDND induces G_2/M arrest and apoptosis in cancer cells possibly due to direct DNA damage or interference with topoisomerase II.*

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Adult T-cell leukemia (ATL) is an aggressive hematological malignancy in which the human T-cell lymphotropic virus type 1 (HTLV-1) has been causally implicated (1). ATL is characterized by rapid resistance to standard chemotherapeutic agents and poor survival, even with hematopoietic stem cell transplantation (HSCT) (2).

The HTLV-1 oncogene-like transactivating protein, Tax, induces cellular immortalization and leukemic transformation through a myriad of mechanisms involving cytokine pathways and cell cycle dysregulation. Tax protein induces expression of cellular growth-related genes, such as granulocyte-macrophage colony stimulating factor (*GM-CSF*) (3, 4), induces interleukin-2 (IL-2) through up-regulation and increased nuclear translocation of nuclear factor kappa B (5), and up-regulates the IL-2 alpha receptor (6). These leukemogenic mechanisms of Tax may be targeted to devise new therapies, such as overcoming the antiapoptotic effects of Tax by the antioxidant pyrrolidinedithiocarbamate (PDTC) (7), or down-regulation of Tax protein to increase susceptibility of leukemia cells to etoposide (8).

Oncogenic unrelated factors associated with chemotherapy resistance include the drug transporter proteins ATP-binding cassette sub-family (CFTR/MRP) member 1 (ABCC1), ATP-binding cassette sub-family G (WHITE) member 2 (ABCG2), and ATP-binding cassette sub-family B (MDR/TAP) member 1 (ABCB1), although these are not specific to leukemia. Calcium channel blocker verapamil and

immunosuppressive agent cyclosporine A have been shown to block ABCB1-mediated efflux of anticancer drugs (9), and other agents reduce cellular glutathione required for ABCC1-mediated transportation of anticancer drugs (10). The relatively high doses required and the toxicity of known resistance-modulating agents necessitate an ongoing search for novel agents (11). In the present study, we report the bioassay-directed identification of a novel cytotoxic agent from the Jamaican plant *Hyptis verticillata* jacq, which is effective against several human hematological and epithelial malignancies, while being having a wide therapeutic index, and able to overcome ABCC1, ABCG2, and ABCB1-mediated resistance *in vitro* at relatively low concentrations.

Materials and Methods

Plant materials and isolation of compound. Aerial parts of *Hyptis verticillata* jacq were collected during the summer of 2007 in Kingston, Jamaica, and voucher specimens were deposited in the herbarium at the University of the West Indies, Mona, Jamaica. Methanolic extracts obtained from the chopped aerial parts of the plant were partitioned between water and diethyl ether. The ether extract was further partitioned between methanol/water (9:1) and *n*-hexane, the aqueous methanol layer subjected to silica gel flash chromatography, and 4-methoxy-9-(3,4,5-trimethoxyphenyl)-8,9-dihydrofuro[3',4':6,7]naphtho[2,3-*d*][1,3]dioxol-6(5*H*)-one (4-MTDND) obtained from fractions eluted with hexane/ethyl acetate (2:3) and purification by octadecyl silane-high performance liquid chromatography (ODS-HPLC). For comparisons, the standard chemotherapeutic agents etoposide and mitoxantrone were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cells. S1T (non-Tax-expressing), K3T (Tax-expressing) and KaT01F (IL-2-dependent) ATL cell lines established in our laboratory were maintained in RPMI-1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 2 mM L-glutamate, and split every 2 to 3 days, corresponding to the log phase of growth; Jurkat, human T-cell lymphoblastoid cell line (kind courtesy of Professor W. C. Greene of the Department of Microbiology and Immunology, Howard Hughes Medical Institute, Duke University Medical Center, Durham, North Carolina, USA) and HL60, human promyelocytic leukemia cell line (obtained from the Hayashibara Cell Bank, Okayama, Japan) were similarly maintained. The human colon adenocarcinoma cell line (SW480) (courtesy of Professor S. Natsugoe, Department of Surgical Oncology and Digestive Surgery, Kagoshima University Hospital, Kagoshima City, Japan), non-small cell lung cancer cell line (A549) (obtained from the Japanese Collection of Research Bioresources, Shinjuku, Japan), human epidermoid carcinoma cells (KB-3-1) or drug-resistant stably transfected with *ABCC1* cDNA (KBABCC1) or Pgp-expressing KBG-2, and erythromyeloblastoid leukemia cells (K562) or drug-resistant stably transfected with *ABCG2* cDNA (K562/ABCG2) were maintained in the same medium, and retrieved from culture flasks with trypsin and washed before use in assays. The KB-3-1 cell line was kindly donated by Professor Shin-ichi Akiyama of the University of Tokushima; KBG-2 and KBABCC1 by Professor Ueda Kazumitsu of Kyoto University; and K562 and K562/ABCG2 by Professor Yoshikazu Sugimoto of Keio University.

Cytotoxicity assay. To assess the cytotoxic effects of 4-MTDND, 1×10^4 cells/well in at least triplicates were incubated in a 96-well flat-bottom plate without or in the presence of serial dilutions of the compound in a humidified incubator at 37˚C. Cells were retrieved after 72 h and (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reagent was added to each well and incubated for 4 hours, followed by dissolution of formazan crystals with 20% sodium dodecyl sulphate, after which plates were left to stand in the dark for at least 2 h before reading the absorbance at 540/630 nm on a microplate reader (Bio-Rad, Hercules, CA, USA). Untreated cells were assigned a value of 100% viability, and viability of treated cells was expressed relatively to that of the untreated controls. The WST-8 assay (Nacalai Tesque, Kyoto, Japan) was used to compare multidrug resistant cells to their controls.

Apoptotic changes in cellular morphology. S1T leukemia cells (1×10^4/ml/well) were incubated without or in the presence of 4-MTDND at different concentrations in a 24-well microplate in a humidified incubator at 37˚C for 48 h. Cells were then retrieved and transferred to glass slides with a cytospin apparatus (Cytospin; Shandon, Amstoor, UK), followed by May-Grünwald-Giemsa staining.

Cell cycle analysis. S1T cells were incubated without or in the presence of 4-MTDND at different concentrations in at least triplicates in a 24-well microplate for 24 hours in a humidified incubator at 37˚C. Retrieved cells were then washed in cold phosphate-buffered saline, and fixed in 100% ice cold ethanol for 30 minutes. Cells were then stained with 50 μg/ml propidium iodide and 100 μg/ml RNase (Sigma-Aldrich), and incubated in the dark at room temperature. Cell cycle analysis was performed on a FACScan after calibration with BD DNA Quality Control kit (BD Bioscience, San Jose, CA, USA) containing chicken erythrocyte nuclei (CEN) and calf thymocyte nuclei (CTN) for linearity and double discrimination, respectively. Data were analysed with ModFit software (Verity Software House, Topsham, ME, USA). Cell cycle regulatory proteins were analyzed by intracellular staining of cells with antibodies to cyclin B1 (Invitrogen, Camarillo, CA, USA) and phospho-cyclin-dependent kinase 1 (cdk1)-pThrpTyr14/15 (MBL, Woburn, MA, USA), followed by secondary staining with fluorescence-labeled anti-mouse or anti-rabbit antibody, respectively. Flow cytometric analysis was performed on a FACScan with Cell Quest software (BD Biosciences).

Western blot analysis. Cells were incubated without or in the presence of 4-MTDND for 48 hours in a humidified incubator at 37˚C. Whole-cell lysates were obtained using cell lysis buffer (Cell Signaling Technology, Beverly, MA, USA) with ethylenediaminetetra-acetic acid-free Protease Inhibitor Cocktail (Thermo Fisher Scientific, Rockford, IL, USA), and separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 4-15% Mini-PROTEAN TGX Precast Gels (Bio-Rad, San Jose, CA, USA). Proteins were transferred to Immun-Blot PVDF Membranes (Bio-Rad) using Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). Membranes were probed after blocking with 1% Casein Blocker in Tris-buffered saline (Bio-Rad) with antibodies to p53 (FL-393; Santa Cruz Biotechnology, Santa Cruz, CA, USA), BAX (N-20, Santa Cruz Biotechnology), and beta-actin (Sigma-Aldrich), as well as with the Apoptosis Antibody Sampler Kit (Cell Signaling Technology) including antibodies to caspase-3, cleaved caspase-3 (Asp175) (5A1E), poly (ADP-ribose) polymerase (PARP), cleaved PARP

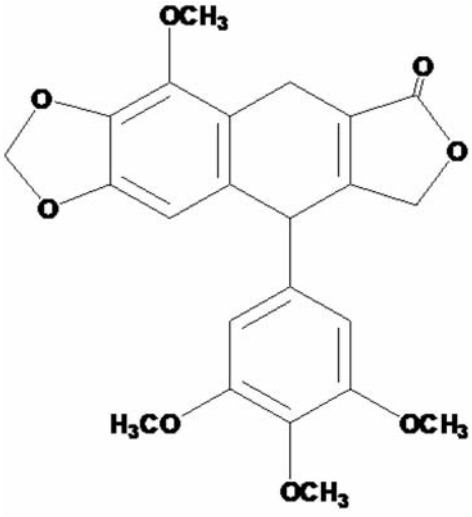


Figure 1. *Chemical structure of 4-methoxy-9-(3,4,5-trimethoxyphenyl)-8,9-dihydrofuro[3',4':6,7]naphtho[2,3-d][1,3]dioxol-6(5H)-one (4-MTDND). Relative molecular weight, 426.16.*

(Asp214) (D64E10), caspase-9, cleaved caspase-9 (Asp330), and anti-rabbit IgG isotype. Secondary staining with horseradish peroxidase-labeled antibody after washing, and the ECL Advance Western Blotting Detection Kit (GE Healthcare, Piscataway, NJ, USA) or Immun-Star HRP Substrate Kit (Bio-Rad) were used for visualization of protein bands according to the manufacturer's instructions.

Results

MTT assay-directed isolation of novel cytotoxic agent. A novel cytotoxic agent was isolated from the methanolic extract obtained from aerial parts of the Jamaican *Hyptis verticillata* jacq plant and identified by nuclear magnetic resonance (NMR) and other chemical analyses as IUPAC name 4-methoxy-9-(3,4,5-trimethoxyphenyl)-8,9-dihydrofuro[3',4':6,7]naphtho[2,3-*d*][1,3]dioxol-6(5*H*)-one, referred to in this article as 4-MTDND (Figure 1). Viability assay-directed isolation also identified several known cytotoxic agents including beta-peltatin – the precursor of chemotherapeutic agent etoposide, hyptinin, and deoxypicropodophyllin. The novel compound, 4-MTDND, exhibited cytotoxic effects towards adult T-cell leukemia cells and other cancer cells, with 50% inhibitory concentration (IC_{50}) as low as 4.7 nM, and exhibited a wide therapeutic index, with a 30- to 40-fold higher IC_{50} of 211 nM towards normal activated peripheral blood mononuclear cells (PBMCs) from a healthy donor (Figure 2A, B). Susceptible cancer cell lines included those of hematological malignancies such as the HTLV-1 Tax oncoprotein-expressing ATL cell line, K3T, as well as the human colon adenocarcinoma cell line SW480, and non-small cell lung cancer cell line A549. IC_{50} data are summarized in Table I. These data indicate that the novel natural product 4-MTDND is cytotoxic towards several hematological and non-hematological cell lines.

Table I. *Cytotoxic effects of 4-MTDND.*

Cell line	Type of malignancy	IC_{50} (nM)
S1T	Tax(–) ATL	7.0
K3T	Tax(+) ATL	9.4
Jurkat	Lymphoblastoid leukemia	4.7
K562	Erythromyeloblastoid leukemia	94.0
HL60	Promyelocytic leukemia	70.0
A549	NSC lung cancer	11.8
SW480	Colon adenocarcinoma	23.6
PBMCs	-	211

IC_{50}: 50% Inhibitory concentration; ATL: adult T-cell leukemia; NSC: non-small cell; PBMCs: activated peripheral blood mononuclear cells.

Effect of 4-MTDND on apoptosis and cell cycle of leukemia cells. To elucidate the mechanism by which 4-MTDND induces cell death, treated and untreated ATL cells were examined by May-Grünwald-Giemsa staining and DNA histogram analysis on flow cytometry. Treatment with 4-MTDND resulted in disruption of cell nuclear material, formation of apoptotic bodies and eventual cell death after 48 hours (Figure 3A), and up-regulation of proapoptotic proteins, including cleavage of PARP, caspase-3 and caspase-9 (Figure 3B). Furthermore, DNA histrogram analyses by propidium iodide staining and flow cytometry after 24 hours of treatment showed a dose-dependent increase in the proportion of S1T leukemia cells in the G_2/M phase of the cell cycle, as well as an increase in the proportion of cells in the sub-G_1 phase (Figure 3C). Similar results were obtained with 4-MTDND treatment of the HTLV1 Tax oncoprotein-expressing ATL cells, K3T, as well as the IL-2-dependent ATL cell line KaT01F (data not shown). Accordingly, permeabilization and intracellular staining with monoclonal antibodies to cyclin B1 and phosphorylated cdk1 and secondary staining with the appropriate fluorescence-labeled antibody revealed a significant decrease in cyclin B1, and an increase in the inactive phosphorylated form of cdk1 after 48 hours' treatment with 4-MTDND (Figure 3D). These data indicate that 4-MTDND exhibits its cytotoxic effects on leukemia cells by inducing apoptosis and promoting G_2/M arrest.

Effect of 4-MTDND on multidrug-resistant cancer cells. Due to the reason that drug resistance remains a significant hurdle in the clinical management of various types of cancer, the effect of 4-MTDND on several multidrug-resistant cancer cell lines was also determined. The KBABCC1 cell line, human epidermoid carcinoma cells stably transfected with *ABCC1* cDNA, was as susceptible to the cytotoxic effects of 4-MTDND as its corresponding non-resistant control cells, KB-

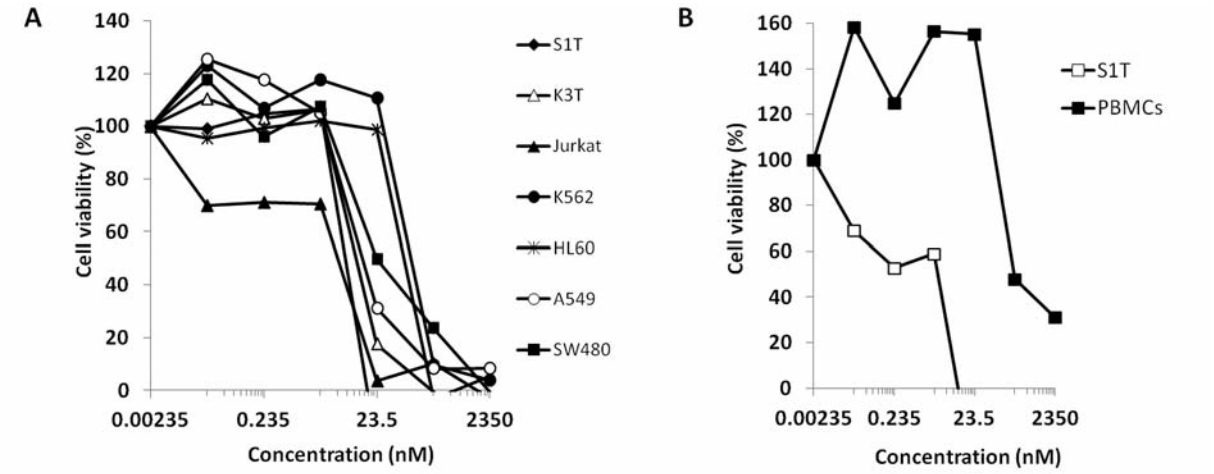


Figure 2. *Cytotoxic effects of 4-MTDND against several cancer cell lines. The 50% inhibitory concentration (IC_{50}) was determined by incubating cancer cells (A) or activated normal peripheral blood mononuclear cells (PBMCs) (B) with serial dilutions of 4-MTDND, and cell viability assessed after 72 hours with (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide and dissolution of formazan crystals with sodium dodecyl sulphate. Viability was expressed relative to the one of untreated cells, which were assigned 100% viability. S1T and K3T are adult T-cell leukemia cell lines; Jurkat, human T-cell lymphoblastoid cell line; K562, a human erythromyeloblastoid leukemia cell line; HL60, human promyelocytic leukemia cell line; A549, non-small cell lung cancer cell line; and SW480, human colon adenocarcinoma cell line.*

3-1 with IC_{50}s of 8.2 and 7.0 nM, respectively (Figure 4A). Furthermore, IC_{50}s of 4-MTDND towards KB-3-1 and KBABCC1 cancer cells were approximately 20-fold and 400-fold lower, respectively, than the IC_{50}s of etoposide towards the same cells (Figure 4A). Similarly, K562/ABCG2 cells showed susceptibility to the cytotoxic effects of 4-MTDND at an IC_{50} of 8.2 nM, approximately 30-fold lower than that of the chemotherapeutic agent mitoxantrone (Figure 4B). The Pgp-expressing KBG-2 cell line was also susceptible to 4-MTDND, with an IC_{50} of 7.7 nM, 110-fold lower than the IC_{50} of etoposide against the same cells. Notably, the IC_{50}s of 4-MTDND towards the three multidrug-resistant cancer cell lines tested, were comparable to the IC_{50} of 4-MTDND towards the non-resistant S1T leukemia cells (Figure 4A-C). The novel cytotoxic agent 4-MTDND is effective against several multidrug-resistant cancer cell lines, and at significantly lower doses than etoposide or mitoxantrone.

Discussion

A novel cytotoxic agent, 4-MTDND, was isolated from the Jamaican folk medicinal plant *Hyptis verticillata* jacq, exhibiting cytotoxic effects through apoptosis induction in a variety of hematological and non-hematological cancer cells, including multidrug-resistant cell lines. This compound exhibited a wide therapeutic index, with cytotoxicity to normal activated PBMCs from a healthy donor that was 30- to 40-fold higher than that towards cancer cells. Multidrug-resistant cancer cells were as susceptible to 4-MTDND as their non-resistant control counterparts, and 4-MTDND was more than 20-fold more cytotoxic to multidrug-resistant cancer cells than available chemotherapeutic agents, etoposide and mitoxantrone. These results indicate that the novel natural plant-derived cytotoxic agent 4-MTDND is cytotoxic towards a variety of cancer cells, and effective against several multidrug-resistant cancer cells.

Treatment with 4-MTDND resulted in morphological changes consistent with apoptosis induction, which was supported by up-regulaton of proapoptotic proteins on Western blot analyses. Normal cellular response to DNA damage includes the up-regulation of the tumor suppressor protein p53, and its activation by ataxia telangiectasia mutated kinase (ATM). Phosphorylation of p53 interferes with binding by its negative regulator oncoprotein. This leads to the activation of the proapoptotic protein, BAX, which enhances mitochondrial membrane permeability to cytochrome *c*. In turn, cytochrome *c* leads to activation of caspase-9 by associating with caspase-9/apoptotic protease-activating factor-1 (APAF-1), and the subsequent activation of caspase-3, and cleavage of PARP, resulting in apoptosis (13, 14). In western blot analyses, 4-MTDND clearly increased BAX and p53 expression, as well as cleavage of caspase-9, caspase-3 and PARP, in a dose-dependent manner consistent with induction of apoptosis due to DNA damage, possibly directly by 4-MTDND or by its interference with topoisomerase II. Furthermore, up-regulation of p53 also affects the cell cycle due to direct activation by ATM or indirectly through checkpoint kinase 2 (chk2). G_2/M arrest, down-regulation of cyclin B1, and increased levels of inactivated phosphorylated cdk1 were evident following 4-MTDND treatment.

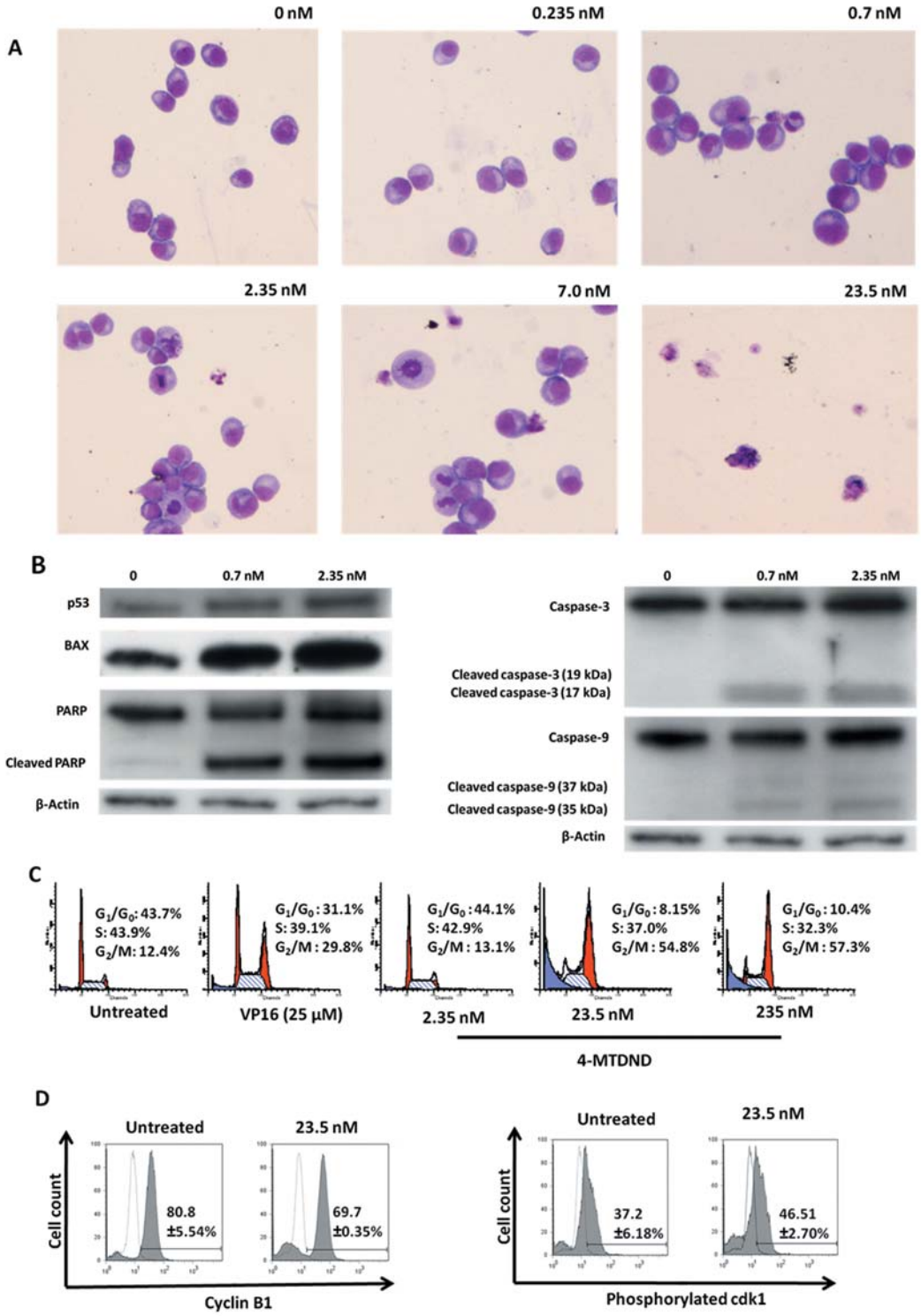
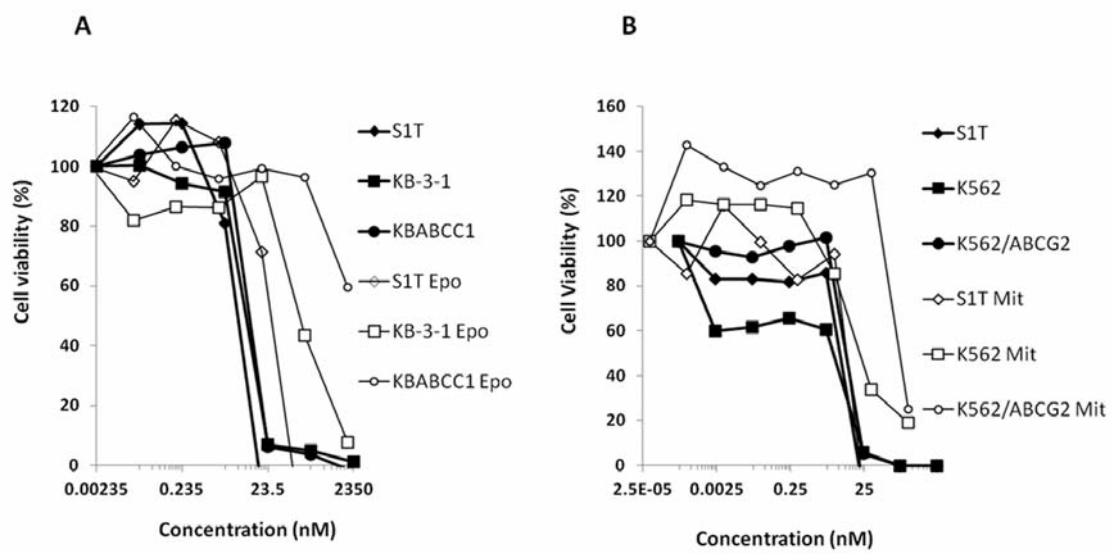
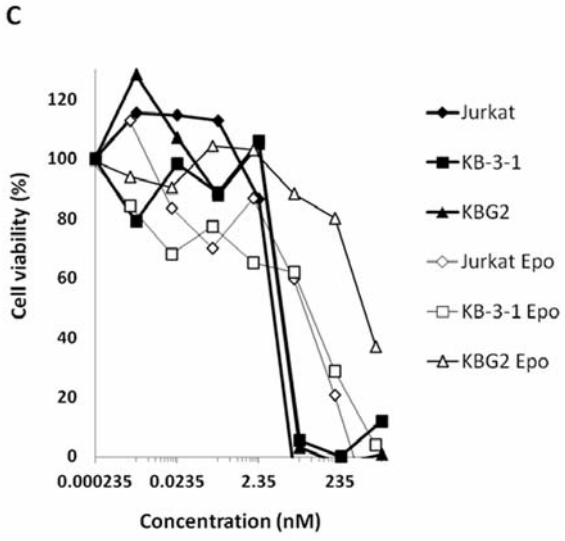


Figure 3. *Apoptosis induction and cell cycle arrest by 4-MTDND. A: May-Grünwald-Giemsa staining of S1T cells without treatment or after incubation with increasing concentrations of 4-MTDND as indicated, illustrating formation of apoptotic bodies and disruption of nuclear material. B: Western blot analyses of apoptosis-related proteins on whole-cell lysates from S1T leukemia cells treated with 4-MTDND at the concentrations indicated. C: G_2/M cell cycle arrest by 4-MTDND in S1T leukemia cells. Cells were incubated for 24 hours without treatment or with 4-MTDND or etoposide, washed in PBS, fixed with ice-cold ethanol, followed by staining with propidium iodide and RNASE A. DNA histograms were generated with Modfit software, with coefficient of variation ranging from 1.92 to 7.05%. Results were obtained from at least three independent experiments and standard deviations are shown. D: Cell cycle regulatory protein expression as assessed by flow cytometry, with means and standard devietions shown.*



Cell line	IC_{50} (nM)	
	4-MTDND	Etoposide
S1T	4.7	30.6
KB-3-1	7.0	118.9
KBABCC1	8.2	3397.8

Cell line	IC_{50} (nM)	
	4-MTDND	Mitoxantrone
S1T	6.3	7.7
K562	4.2	15.3
K562/ABCG2	8.2	229.7



Cell line	IC_{50} (nM)	
	4-MTDND	Etoposide
Jurkat	4.7	29
KB-3-1	7.7	36
KBG2	7.7	850

Figure 4. *Cytotoxic effects of 4-MTDND compared to etoposide (A), and mitoxantrone (B) on carcinoma cell lines and corresponding multidrug-resistant cell lines. KB-3-1, human epidermoid carcinoma cell line; KBABCC1, KB-3-1 cells stably transfected with ABCC1 cDNA; K562, human erythromyeloblastoid leukemic cell line; K562/ABCG2, K562 cells stably transfected with ABCG2 cDNA. C: Effect of 4-MTDND compared to etoposide on KBG-2, P-glycoprotein-expressing multidrug-resistant carcinoma cells. Epo, Etoposide; Mit, mitoxantrone; S1T, adult T-cell leukemia cell line; Jurkat, human T-cell lymphoblastoid cells. IC_{50} data are summarized in the corresponding table below each graph.*

The novel cytotoxic agent, 4-MTDND, shares a lignan carbon backbone with the previously described agents 4-demethyldesoxypodophyllotoxin and beta-peltatin, also isolated from *Hyptis verticillata* (15). Unlike these podophyllotoxins, which generally have broad nonspecific cytotoxicity, 4-MTDND exhibits significantly lower toxicity towards normal human PBMCs from healthy donors, and is active against several types of hematological and epithelial cancer cells. Further studies with *in vivo* cancer models, and experiments elucidating the mechanism of overcoming multidrug resistance would advance this novel agent as a useful anticancer drug.

Conflict of Interest Statement

Y. White, T. Hamada, M. Nakashima and N. Arima are applying for a patent on 4-MTDND. The other Authors declare that there are no conflicts of interest.

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