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Cell cycle arrest and proapoptotic effects of the anticancer cyclodepsipeptide serratamolide (AT514) are independent of p53 status in breast cancer cells☆

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AT514, serratamolide
HPLC, high performance liquid chromatography
KF, Kahalalide F
MALDI, matrix-assisted laser desorption/ionization
MPLC, medium pressure liquid chromatography
MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide
NMR, nuclear magnetic resonance
RT-PCR, real-time quantitative polymerase chain reaction
TOF, time of flight

ABSTRACT

In a search for new anticancer agents, we have identified serratamolide (AT514), a cyclodepsipeptide from *Serratia marcescens* 2170 that induces cell cycle arrest and apoptosis in various cancer cell lines. A cell viability assay showed that the concentrations that cause 50% inhibition (IC_{50}) in human cancer cell lines range from 5.6 to 11.5 μM depending on the cell line. Flow cytometry analysis revealed that AT514 caused cell cycle arrest in G_0/G_1 or cell death, depending on the cell type and the length of time for which the cells were exposed to the drug. Subsequent studies revealed that AT514-induced cell death is caused by apoptosis, as indicated by caspases activation (8, 9, 2 and 3) and cleavage of poly (ADP-ribose) polymerase (PARP), release of cytochrome *c* and apoptosis inducing factor (AIF) from mitochondria, and the appearance of apoptotic bodies and DNA laddering. Alterations in protein levels of Bcl-2 family members might be involved in the mitochondrial disruption observed. AT514 induced p53 accumulation in wild-type p53 cells but cell death was observed in both deficient and wild-type p53 cells. Our results indicate that AT514 induces cell cycle arrest and apoptosis in breast cancer cells irrespectively of p53 status, suggesting that it might represent a potential new chemotherapeutic agent.

☆ AT514: under patent PCT WO2004/031130 A1.

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1. Introduction

Depsipeptides are bio-oligomers composed of hydroxy and amino acids linked by amide and ester bonds. Many depsipeptides show very promising biological activities, including anticancer, antibacterial, antiviral, antifungal and anti-inflammatory properties [1]. In particular, cyclodepsipeptides (cyclic depsipeptides), such as Didemnin (A–E), Kahalalide F (KF), and FR901228 are currently under active anticancer research specifically focused on identifying their mechanism of action [2–4].

Apoptosis is a tightly regulated form of cell death in which cells actively participate in their own destruction. Drug-induced apoptosis is mainly initiated by either the activation of cell surface receptors or by directly targeting mitochondria [5]. Bcl-2 family members are responsible for integrating the apoptotic stimulus at the mitochondrial level and are involved in this cascade by either promoting (Bax, Bid) or preventing (Bcl-2, Bcl-X_L) mitochondria-dependent apoptosis [6]. This process is accompanied by the activation of aspartate-specific proteases called caspases [7]. In the receptor pathway, the initiator caspase 8 is activated, whilst in the mitochondrial pathway, cytochrome c is released into the cytoplasm and in turn caspase 9 is activated by forming a complex with Apaf-1. The tumor suppressor protein p53 or caspase 2 have been reported to activate the second pathway in response to DNA damage. Both pathways ultimately activate the effector caspases 3 or 7, which cleaves a wide range of substrates, leading to the morphological and biochemical changes that are the hallmarks of apoptosis [8]. Cells undergoing apoptosis shrink and lose their normal intercellular contacts and subsequently exhibit cytoplasmic and chromatin condensation and internucleosomal cleavage of DNA. In the final stages, cells become fragmented into small apoptotic bodies, which are then eliminated by phagocytosis.

Malfunctions of apoptosis can have health implications, as in the case of cancer. Resistance acquired by tumor cells after conventional chemotherapy is a major problem in cancer treatment. Thus, there is a need to develop new anticancer agents and therapeutic regimens for successful treatment. Consequently, the fact that apoptosis is a precisely regulated process that is frequently altered in tumor cells makes it a desirable target for the induction of cell death in cancer cells [9]. Indeed, it has already been described that most anti-tumoral agents kill tumor cells by activating apoptosis [10].

In our laboratory, during a search for new potential anticancer agents, we observed the presence of serratamolide (AT514) in cultures of the bacterial strain *Serratia marcescens* 2170 in the stationary growth phase. AT514 is a hydrophobic cyclic depsipeptide and is the main component of cell-wall lipids. It confers to bacteria wetting and spreading properties, reducing the surface tension of water and therefore increasing its adhesion to solid surfaces [11], and contributes to the virulence of *S. marcescens* [12]. In this view, given the lack of studies related to cancer therapy using AT514, we have investigated whether this molecule has cytotoxic activity against human cancer cells. We show that AT514 arrests the cell cycle at G_0/G_1 and induces apoptosis in human breast cancer cell lines. Furthermore, we describe the molecular apoptotic events triggered by this new symmetrical cyclic depsipeptide as well as its independence of the tumor suppressor protein p53. Taken together, the results of this study indicate that AT514 might represent a potential new anticancer agent since it effectively induces apoptosis in breast cancer cells irrespectively of p53 status.

2. Materials and methods

2.1. Purification and characterization of serratamolide (AT514)

AT514 (serratamolide), cyclo[(3R)-3-hydroxydecanoyl-L-seryl-(3R)-3-hydroxydecanoyl-L-seryl], was extracted by shaking *Serratia marcescens* 2170 cells with a mixture of methanol and 1 N HCl (24:1). After centrifugation (6800 × *g* for 15 min), the solvent of the supernatant was evaporated under vacuum. Atmospheric pressure liquid chromatography of the extract was performed on silica gels (pore size 60 Å) with chloroform:methanol (6:4) as solvents. The eluted fractions containing the two major products (further characterized as prodigiosin and serratamolide) were pooled and the chloroform/methanol extract was vacuum evaporated, redissolved in H_2O and lyophilized. The sample mixture was analyzed by electrospray ionization/mass spectrometry (ESI-MS) using a VG-Quattro® triple quadrupole mass spectrometer (Micromass, VG-Biotech, UK) and by matrix-assisted laser desorption (MALDI) using a Voyager delayed extraction (DE) time of flight (TOF) mass spectrometer (PerSeptive Biosystems, Framingham, USA). The product with a molecular weight (*m*/*z* 515) consistent with that expected for serratamolide was isolated from the pigmented mixture by two consecutive medium pressure liquid chromatography (MPLC) steps. MPLC was performed on a system containing a CFG® Prominent/Duramat pump, a variable wavelength LKB® Bromma 2158 UVICORD SD detector (206 nm filter), a Gilson FC 205 collector, and a Pharmacia-LKB REC 101 register. In the first step, a Lichroprep C_8 reversed-phase glass column (20 cm × 4 cm) was used with a 0–100% B linear gradient (A: 0.01 M ammonium acetate pH 7.0; B: 100% acetonitrile), 1000 ml total volume at a flow rate 240 ml/h. The eluted fractions were analyzed by HPLC (high performance liquid chromatography) on a Shimadzu LC-6A instrument with a Nucleosil® C_{18} analytical reversed-phase column (25 cm × 0.4 cm) and by MALDI-TOF. Fractions containing serratamolide were pooled, vacuum evaporated, and lyophilized. The second MPLC purification step was performed using a C_{18} reversed-phase glass column (26 cm × 2.5 cm) and a 35–55% B linear gradient (A: H_2O 0.045% trifluoroacetic acid; B: CH_3CN 0.036% trifluoroacetic acid), 800 ml total volume at a flow rate 120 ml/h. The eluted fractions were analyzed by MALDI-TOF and HPLC. A pure product (96% purity determined by HPLC) was obtained after pooling, vacuum evaporation, and lyophilization of the desired fractions. Quantification was performed by amino acid analysis on a Beckman 6300 automatic analyzer with a sulfonated-polystyrene column (25 cm × 0.4 cm, Beckman 7300/6300). The product was characterized by mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy (Fig. 1A). Exact mass determination was performed by chemical ionization (CI) with methane in an AutoSpec

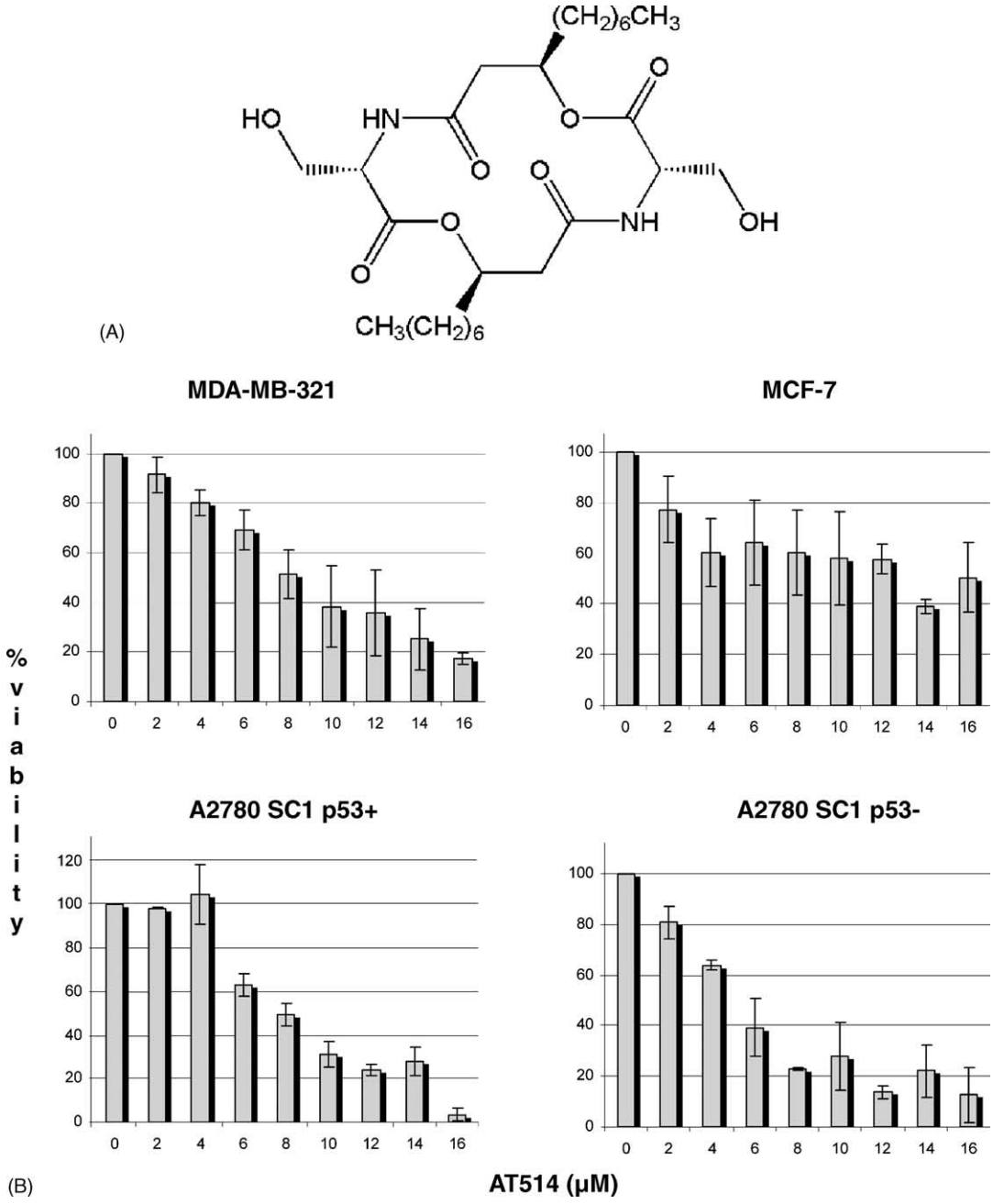


Fig. 1 – (A) Structure of serratamolide (AT514). (B) AT514 effect on cell viability after 24 h of drug exposure (from 2 to 16 μM) was analyzed by MTT assay.

magnetic sector mass spectrometer N/S-X134: *m/z* 515.335020 $(M + H)^+$. ESI-MS: *m/z* 515.2 $(M + H)^+$. MALDI-TOF: *m/z* 515.8 $(M + H)^+$, 537.8 $(M + Na)^+$, 553.8 $(M + K)^+$. ($C_{26}H_{46}N_2O_8$) requires 514.3254 (MWmonoisotopic) ^1H NMR (CD_3OH, 500 MHz, p.p.m.): 7.88 (d, 1NH), 5.28 (m, 1H), 4.47 (m, 1H), 4.07 (dd, 1H), 3.82 (dd, 1H), 2.67 (dd, 1H), 2.33 (dd, 1H), 1.66 (m, 2H), 1.3 (m, 10H), 0.89 (t, 3H). The results were in agreement with previous NMR studies [13].

2.2. *Cell lines and culture conditions*

Breast cancer cell lines MDA-MB-231 (cells with mutated p53) and MCF-7 (cells with wild-type p53) were purchased from ATCC (Rockville, MD) and cultured in DMEM:HAM F12 (1:1). The ovarian carcinoma clones from the A2780 cells were a generous gift from Dr. Karran (Cancer Research, London, UK) and were previously described [14]; the A2780 SC1 p53+ retain an intact p53 response while in the A2780 SC1 p53−, the p53 response was inhibited by introducing a dominant negative mutant p53 (p53val135). Both were cultured in DMEM. Media were purchased from Biological Industries (Beit Haemek, Israel) and supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 50 mg/ml gentamycin (GIBCO BRL, Paisley, UK). Cells were grown in a humidified atmosphere of air containing 5% CO_2 at 37 °C.

2.3. Cell viability assay

Cell viability was determined using the MTT assay [15]. Cells were plated in triplicate wells (2×10^4 cells/well) in 100 μl of growth medium in 96-well plates and treated with increasing concentrations of AT514 (2–16 μM) or drug diluent (DMSO). Adherent cell lines were plated 24 h before treatment at a concentration of 1×10^4 cells/well. After 24 h incubation with AT514, 10 μM of MTT (Sigma Chemical Co., St. Louis, MO) was added to each well for an additional 4 h. The blue MTT formazan precipitate was then dissolved in 100 μl of *iso*-propanol: 1N HCl (24:1). The absorbance at 570 nm was measured on a multiwell plate reader. Cell viability was expressed as a percentage of control and IC_{50} represents the concentration of drug causing 50% inhibition of the increase in absorbance compared with control cells. Data are shown as the mean value ± S.E.M. of three independent experiments.

2.4. Assessment of cell cycle arrest

Cells (1×10^6) were treated with 4, 8, and 12 μM AT514 for 24 h and fixed in 70% ethanol at −20 °C overnight. Then, they were washed in PBS and incubated with 25 μl of 1 mg/ml propidium iodide (Bender MedSystems Inc., Burlingame, CA) and 5 μl of 10 mg/ml DNase free RNase (Boehringer Mannheim, Mannheim, Germany) for 30 min at 37 °C in the dark. Fluorescence was measured by flow cytometry on a FACSCalibur fitted with a 488 nm Ar laser, and data were analyzed using CellQuest Pro software (Becton Dickinson, San Jose, CA) and ModFit LT cell cycle analysis software (Verity software, Topsham, ME).

2.5. Analysis of internucleosomal DNA fragmentation

Cells (5×10^5 cells/ml) were treated with AT514 for 24 h. After washing the cells in PBS, they were lysed in 400 μl of lysis buffer (10 mM Tris–HCl pH 7.4, 1 mM EDTA, 0.2% Triton X-100) for 15 min at 4 °C. Then, cell lysates were centrifuged at 14 000 × *g* for 15 min to separate low molecular weight DNA from intact chromatin, and supernatants were treated with 0.2 mg/ml proteinase K (Sigma Chemical Co.) in a buffer containing 150 mM NaCl, 10 mM Tris–HCl pH 8.0, 40 mM EDTA, and 1% SDS, for 4 h at 37 °C. After two extractions with phenol:chloroform:*iso*-amylalcohol (25:24:1), the aqueous supernatants were precipitated with two volumes of ethanol plus 140 mM NaCl at −20 °C overnight, and recovered by centrifugation at 14 000 × *g* for 15 min at 4 °C. The DNA pellets were then washed twice in cold 70% ethanol, air-dried, and resuspended in 15 μl of TE (10 mM Tris–HCl pH 8.0, 1 mM EDTA) and treated with DNase free RNase for 1 h at 37 °C. Finally, DNA was analyzed by electrophoresis on a 1.2% agarose gel.

2.6. Hoechst staining

Cell morphology was evaluated by fluorescence microscopy following Hoechst 33342 DNA staining (Sigma Chemical Co.). Cells (2×10^5 cells/ml) were incubated in the absence (control cells) or presence of AT514 for 24 h. They were then washed in PBS and resuspended in PBS containing 2 μg/ml Hoechst 33342 and incubated for 30 min at 37 °C in the dark. After incubation, cells were washed in PBS and examined with a Carl Zeiss Jena microscope and photographed with an Olympus DP11 digital camera.

2.7. Western blot

Cells (2×10^5 cells/ml) were exposed to 4, 8, or 12 μM AT514 for 24 h. They were then washed in PBS prior to addition of a lysis buffer (85 mM Tris–HCl pH 6.8, 2% SDS, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 0.1 mM phenylmethanesulfonyl fluoride). For the measurement of cytochrome *c* and AIF release from mitochondria, lysis buffer (250 mM sucrose, 1 mM EDTA, 0.05% digitonin, 25 mM Tris pH 6.8, 1 mM dithiothreitol, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, 100 μM phenylmethanesulfonyl fluoride) was used for 30 s, lysates centrifuged at 12000 × *g* at 4°C for 3 min and the supernatants (cytosolic extract) were separated from pellets (fraction that contains mitochondria). In all cases, 50 μg protein extracts were separated by SDS-PAGE on a 15% polyacrylamide gel and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Blots were blocked in 5% dry milk diluted in TBS-T (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20) for 1 h and then incubated overnight with primary antibodies. Antibodies were obtained from the following sources: cleaved caspase 3 (Cat#9661), cleaved caspase 7 (Cat#9491) and anti-Bid (Cat#2002 and 2003) were from Cell Signaling Technology (New England Biolabs, Hertfordshire, UK); anti-PARP (Cat#sc-7150), anti-procaspase 2 (Cat#sc-625), and anti-Bcl-X_L (Cat#sc-634) were from Santa Cruz biotechnologies (Santa Cruz, CA); anti-caspase 8 (Cat#559932) and anti-cytochrome *c* (Cat#556433) were from Pharmingen (BD biosciences, Palo Alto, CA); anti-Bcl-2 (Cat#OP60T) and anti-AIF (Cat#PC536) were from Oncogene Research Products (Boston MA); anti-p53 (Cat#MS-186-P1) was from Neomarkers (Fremont, CA); anti-Bax (Cat#AHP471) was from Serotec Ltd. (Oxford, UK); and anti-caspase 9 (Cat#05-572) was from Upstate (Lake Placid, NY). All primary antibodies were used according to the manufacturer's instructions. Antibody binding was detected using a secondary antibody conjugated to horseradish peroxidase (Biorad, Hertfordshire, UK) and the ECL detection kit (Amersham, Buckinghamshire, UK). Protein bands were quantified with the image analysis software program Phoretix 1-D advanced. Results were presented as normalized fold changes respect control. Normalization has been done using vinculin as a loading control.

2.8. Gene expression analysis

Cells (2×10^5 per ml) were treated with 0 (control), 4, 8, or 12 μM AT514. Total RNA extraction was performed using Ultraspec™ RNA (Biotex Laboratories, Texas, USA). The RNA pellet was washed twice in 75% ethanol, dissolved in H_2O, and cDNA synthesis was performed using random hexamers and MuLV reverse transcriptase according to the manufacturer's instructions (Applied Biosystems, Warrington, UK). The final concentration of cDNA was 1 μg in 50 μl. Each cDNA sample was analyzed for expression of Bcl-2 family members using the fluorescent TaqMan 5′ nuclease assay. Oligonucleotide primers Bcl-2 (Cat#Hs00153350_m1), Bax (Cat#Hs00180269_m1), Bcl-X_L (Cat#Hs00236329_m1), and GAPDH (Cat#Hs99999905_m1), and

probes were initially designed and synthesized as Assay-on-Demand Gene Expression Products (Applied Biosystems). The 5′ nuclease assay PCRs were performed using the ABI PRISM 7700 Sequence Detection System for thermal cycling and real-time fluorescence measurements (RT-PCR) (Applied Biosystems). Each 50 μl reaction consisted of 1X TaqMan Universal PCR MasterMix (PE Biosystems), 1X Assay-on-Demand mix containing forward primer, reverse primer, and TaqMan quantification probe (Applied Biosystems), and 100 ng cDNA template. Reaction conditions comprised an initial step of 92 °C for 10 min, then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The levels of Bcl-2 family members obtained were normalized by mRNA expression of GAPDH. The relative mRNA expression was then presented in relation to the control. Data were analyzed using "Sequence Detector Software" (SDS Version 1.9, Applied Biosystems).

3. Results

3.1. *Effect of AT514 on cell viability*

The antiproliferative effect of AT514 was determined using the MTT reduction assay in breast and ovarian tumor cell lines. Fig. 1B shows the AT514 effects on cell viability after 24 h of drug exposure ranging from 0 to 16 μM AT514. Cell viability significantly decreased in all cancer cells. In the breast cancer cell lines MCF-7 and MDA-MB-231, IC_{50} values were 11.5 ± 0.9 and 9.6 ± 1.1 μM, respectively. However, whereas MDA-MB-231 cell viability decreased more than 80%, MCF-7 viability only decreased 50% at the same doses, which could be explained not by cell death but by a cell cycle blockade. Both ovarian cancer cell lines, A2780 SC1 p53+ (p53 wild type) and A2780 SC1 p53− (in which the p53 response was inhibited by introducing a dominant negative mutant p53 (p53val135)) showed a marked decrease in cell viability. Their IC_{50} values were 8.8 ± 0.6 and 5.6 ± 0.4 μM, respectively, being wild-type p53 cells less sensitive than their mutated p53 counterparts.

3.2. *AT514 induces cell cycle arrest and delayed cell death in MCF-7*

Since AT514 induced a decrease in MCF-7 cell viability of approximately 50%, we next investigated whether this effect could be due to cell cycle arrest. Thus, we analyzed cell cycle progression by flow cytometry using propidium iodide in MCF-7 cells exposed to doses of AT514 ranging from 4 to 12 μM for 24 h. Fig. 2A shows a concentration-dependent accumulation

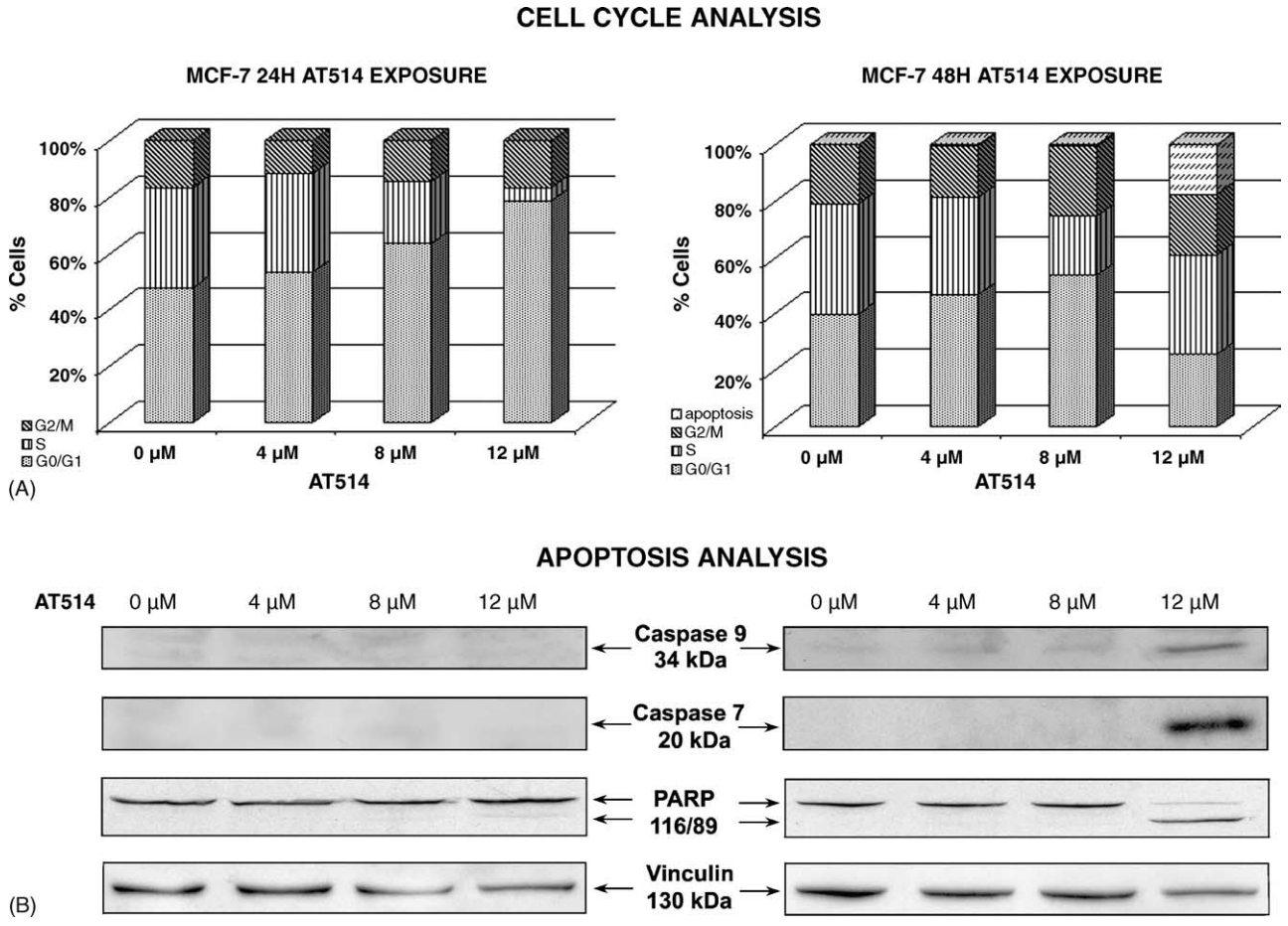


Fig. 2 – Cytostatic and cytotoxic effects of AT514. (A) AT514 induces cell cycle arrest or apoptosis depending on length of exposure in MCF-7 cells. Cells were treated with 4, 8, and 12 μM AT514 for either 24 or 48 h, incubated with propidium iodide, and analyzed by flow cytometry. (B) Apoptosis induction was analyzed by the appearance of caspase activation and PARP cleavage by western blot. Vinculin is shown as a loading control.

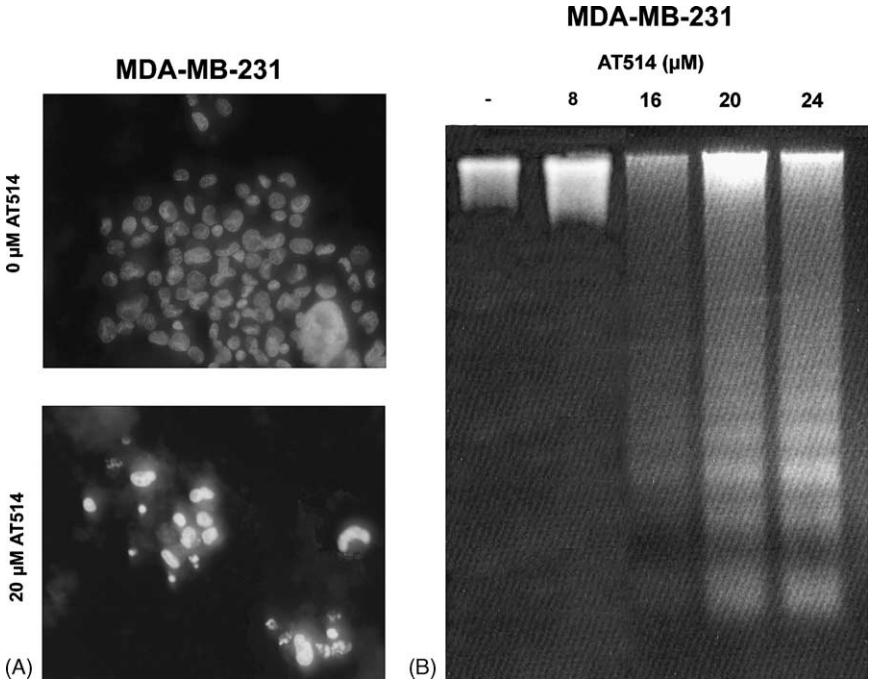


Fig. 3 – AT514 induces apoptosis in MDA-MB-231 cells after 24 h AT514 treatment. (A) Hoechst staining was used to assess nuclear condensation. (B) DNA fragmentation was visualized by analysis of DNA laddering.

of cells in G_0/G_1 (from 47.5 ± 5.5% to 78.9 ± 1.0%), whilst the percentage of cells in S phase decreased sharply. Nevertheless, after incubation with AT514 for 48 h, induction of cell death by apoptosis was detected (21 ± 4.6% at 12 μM AT514), as shown by the appearance of active caspases 9 (34 kDa) and 7 (20 kDa) and the cleaved form of the caspase substrate PARP (89 kDa) (Fig. 2B). Hence, in this cell type, the effect of AT514 depends upon the length of exposure, blocking cell cycle progression after short periods and inducing cell death after longer exposure.

3.3. *AT514-induced apoptosis*

In the other cell types, AT514 caused more than 80% decrease in cell viability hence it was due to cell death. In order to confirm that AT514-induced cell death was caused by apoptosis, classic morphological features of apoptosis were analyzed. The presence of nuclei condensation and apoptotic bodies was assessed using nuclear staining with Hoechst 33342 and an analysis of DNA laddering was performed to reveal internucleosomal DNA fragmentation. Both of these markers of apoptosis were present in AT514-treated MDA-MB-231 cells (Fig. 3A and B, respectively).

3.4. *Caspase activation by AT514*

To evaluate whether apoptosis induced by AT514 was accompanied by caspase activation, MDA-MB-231 cells were exposed to 4, 8, and 12 μM AT514 for 24 h (IC_{25}, IC_{50}, and IC_{75}, respectively) and immunoblotting studies were then performed (Fig. 4). Levels of the inactive form (procaspase) of the initiators caspase 8 and caspase 2 (55/50 and 48 kDa, respectively) decreased significantly at the higher dose, while cleaved caspase 8 (40/36 kDa) gradually appeared. Levels of the active forms of the mitochondrial pathway initiator caspase 9 (34 kDa) and the effector caspase 3 (20 kDa) also increased. This effect was especially marked at higher doses. Examination of the caspase substrate PARP (116 kDa) after AT514

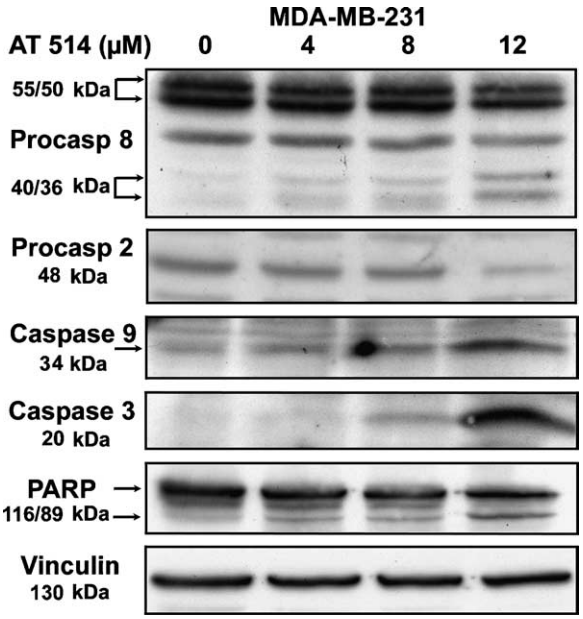


Fig. 4 – Caspase activation and PARP degradation in MDA-MB-231 cells after AT514 exposure. Cells were incubated with 4, 8, and 12 μM AT514 for 24 h and immunoblotting was performed for the inactive proform of caspases 8 and 2, the active form of caspases 9 and 3, and the caspase substrate PARP. Vinculin is shown as a loading control.

treatment showed accumulation of the cleaved product of this protein (89 kDa). Thus, caspases are clearly activated after exposure to AT514, especially at higher doses.

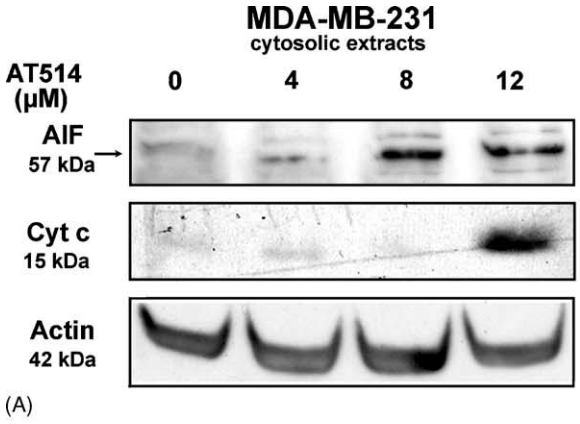
3.5. *Mitochondrial membrane disruption after AT514 treatment*

To further study key events in the apoptotic process, the release of apoptogenic factors, such as cytochrome c and AIF, from mitochondria into the cytosol was analyzed by Western blot (Fig. 5A). Cytochrome c and AIF protein levels were determined in the cytosolic fraction of samples treated with AT514. AIF levels showed a dose-dependent increase in response from 4 to 12 μM AT514. In contrast, cytochrome c was only detectable at higher doses of AT514, suggesting that apoptosis induced at lower doses might be mediated by AIF rather than cytochrome c.

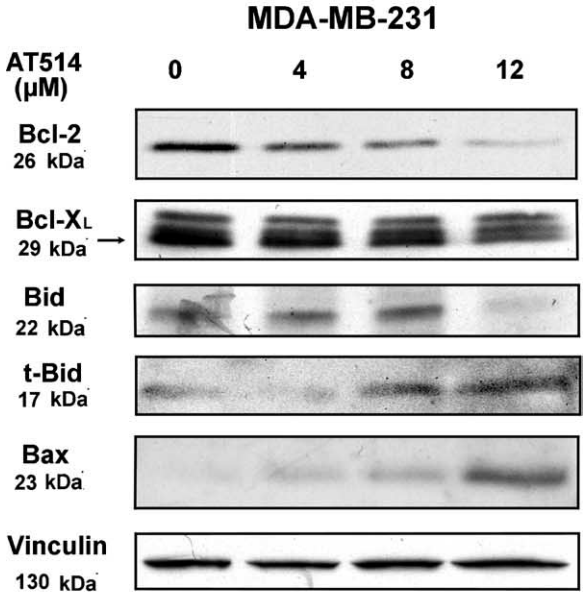
As disruption of mitochondria is mediated by Bcl-2 family members during mitochondria-mediated apoptosis, their protein and mRNA levels were analyzed by immunoblotting and RT-PCR respectively. Levels of the antiapoptotic proteins Bcl-2 and Bcl-X_L decreased in a concentration dependent manner, while significant levels of the proapoptotic Bax protein appeared at 12 μM (Fig. 5B). The proapoptotic form of Bid (t-Bid 17 kDa) increased as disappearance of the large form (22 kDa) was observed, especially at the higher dose. mRNA levels were not significantly affected by AT514 treatment (Fig. 5C), suggesting that Bcl-2 family members are modified at the translational rather than at the transcriptional level. These AT514-induced changes may be involved in the release of apoptogenic factors from the mitochondria.

3.6. *AT514 effects are independent of p53 status*

p53 is a protein able to induce either cell cycle arrest or apoptosis in response to stress-induced DNA damage. AT514 effects were observed in both, wild type p53 (p53+) and mutant p53 (p53−) cell lines, indicating that AT514 acts independently of p53 status. However, in order to study whether exists a role for p53 on the different effects triggered by AT514, MDA-MB-231 cells (cell line with p53−), which underwent apoptosis, and MCF-7 cells (cell line with p53+), which underwent cell cycle arrest, were further analyzed. As expected, the levels of p53 protein were not increased by AT514 treatment in MDA-MB-231 (Fig. 6A). However, p53 levels were also unaltered in MCF-7 cells at the time and doses that AT514 induced cell cycle arrest, but not when it provoked apoptosis (at 12 μM for 48 h) (Fig. 6B). Moreover, p53 response to AT514 treatment was studied in wild type p53 (p53+) A2780 SC1 cells and mutated p53 (p53−) A2780 SC1 cells. IC_{25}, IC_{50} and IC_{75} concentrations were used and p53 accumulation was observed only in A2780 SC1 p53+ cells (almost two folds increase) but not in the p53 deficient clone, as expected (Fig. 6C). Although both of them underwent cell death at the higher dose (Fig. 1B), A2780 SC1 p53+ cells were less sensitive to the drug than their mutated p53 counterparts. The accumulation of functional p53 protein might be the reason why A2780 p53+ detect and react to drug damage in a different way than A2780 p53− cells. Altogether, p53 appears not to be necessary in the cellular response to AT514 exposure although its accumulation is induced by the



(A)



(B)

(C)

GENE EXPRESSION MODIFICATIONS BY RT-PCR

gene	μM AT514	Normalized change folds	S.E.M.
bcl-2	0	1	0,48
	4	1,06	0,38
	8	0,92	0,37
	12	0,95	0,47
bax	0	1	0,42
	4	1,05	0,39
	8	0,70	0,31
	12	0,61	0,24
bcl-X_L	0	1	0,58
	4	0,96	0,46
	8	0,79	0,21
	12	0,81	0,39

Fig. 5 – Involvement of Bcl-2 family members in MDA-MB-231 mitochondrial disruption. Cells were treated with 4, 8, and 12 μM AT514 for 24 h. (A) Cytosolic extracts were isolated in order to analyze the appearance of mitochondrial apoptogenic factors such as AIF and cytochrome c by Western blotting. Actin is shown as a loading control. (B) Total extracts were used to analyze Bcl-2, Bcl-X_L, Bid, t-Bid and Bax protein levels after AT514 treatment. Vinculin is shown as a loading control. (C) Bcl-2, Bax, and Bcl-X_L gene expression changes analyzed by real-time PCR. The values obtained were normalized using mRNA expression of GAPDH.

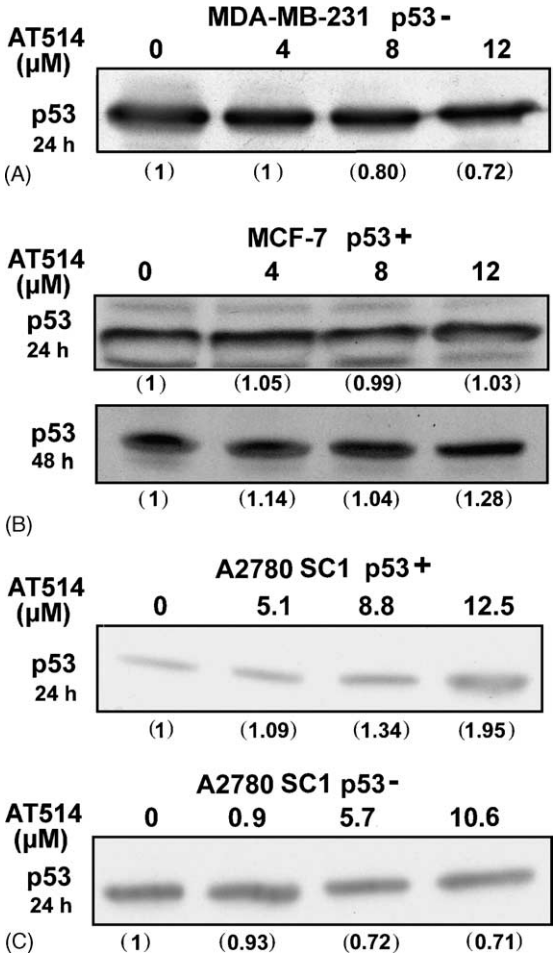


Fig. 6 – Analysis of p53 protein accumulation after AT514 treatment. Cells, p53 mutated (p53−) or p53 wild type (p53+), were incubated with increasing concentrations of AT514 for 24 h or 48 h and p53 protein levels were analyzed by Western blot. Quantification of the bands was performed and normalized change folds respect non-treated cells are shown in brackets. Normalization has been done using vinculin as a loading control.

AT514 cytotoxic effect in those cells with functional p53, but not when AT514 induces cell cycle arrest.

4. Discussion

The induction of apoptosis, or programmed cell death, is thought to be one of the most interesting therapeutic strategies with which to specifically target cancer cells [16]. The limited efficacy of current conventional chemotherapy treatments necessitates the development of new therapeutic agents. The emergence of new anticancer compounds from natural sources with advantageous properties and novel mechanisms of action is continuous, and offers a promising future in the battle against cancer. The cyclodepsipeptides are a broad family of natural products characterized by the presence of at least one ester linkage [1]. Many depsipeptides exhibit a diverse range of biological activities, including antibiotic, antifungal, immunosuppressant or anti-inflammatory and antitumoral effects. Many of the cyclodepsipeptides discovered so far have remarkable in vitro and in vivo anticancer properties against a wide range of tumoral cell lines and some of them are currently undergoing clinical trials in humans [17]. In this study, we have provided the first evidence that the ciclodepsipeptide serratamolide (AT514) induces cell cycle arrest and cell death in various cancer cells. Cell death mediated by AT514 caused caspase activation and induced morphological features typical of apoptosis. This is consistent with the characterization of other depsipeptides, such as FR901228 and IC101, as proapoptotic agents [18–20], a feature shared by almost all currently used chemotherapeutic agents.

Most cytotoxic agents, irrespective of their primary targets, are now thought to kill cells predominantly through the induction of mitochondrial modifications [8,21]. Thus, members of the Bcl-2 family constitute a group of proteins that play important roles in apoptosis regulation [21]. Among the various Bcl-2 homologs identified to date, Bcl-2, Bcl-X_L, and Bax represent the best-characterized members [22]. In this study, we found that AT514 induces downregulation of Bcl-2 and Bcl-X_L and upregulation of Bax in breast cancer cells. Interestingly, real-time PCR analysis showed that AT-514 treatment did not result in changes in Bcl-2, Bcl-X_L, or Bax mRNA levels, suggesting that the effects of the ciclodepsipeptide on this protein family occur at a translational level. Bcl-X_L expression has very recently been proposed to serve as a molecular target for anticancer therapy. Thus, new anticancer agents currently under investigation such as 2,3-DCPE [23] are able to downregulate Bcl-X_L expression, resulting in mitochondria-mediated cell death. Similarly, in the present study we are also able to observe a Bcl-X_L decrease after AT514 treatment, which might be involved in the mitochondrial disruption. However, downregulation of Bcl-X_L is not observed in apoptosis induced by 5-fluouracil or paclitaxel, suggesting that apoptosis itself does not result in downregulation of Bcl-X_L [23]. Another proapoptotic member of the Bcl-2 family is t-Bid. As we have shown here, after treatment with 12 μM AT514 the inactive precursor bid (~22 kDa) almost disappears and the truncated form (t-Bid) is generated. This process may mediate Bax translocation to the mitochondria and cause the release of cytochrome *c* [24]. Similar to our results, t-Bid and the initiator caspase 2, which can activate apoptosis by the intrinsic pathway in response to DNA damage, do actively participate in the apoptotic process induced by other depsipeptides such as FR901228 [25].

AIF, a resident protein of the inter-mitochondrial space, has been implicated as a crucial early effector of apoptosis in a caspase-independent process [26]. We show how AIF was released from mitochondria in a dose-dependent manner starting at 4 μM (IC_{25}) AT514, while cytochrome *c* and caspase activation were not detected until 12 μM (IC_{75}) AT514. Moreover, flow cytometry studies at 4 μM showed no cell cycle arrest (data not shown). These observations suggest that in the presence of low AT514 concentrations AIF released from the mitochondria triggers caspase-independent apoptosis. When we increased the amount of the drug, cytochrome *c* appeared in the cytosol and triggered caspase-dependent apoptosis.

This is corroborated by the caspase activation that was observed at higher doses. The ability to induce cell death both by the caspase-independent and the caspase-dependent apoptotic pathway is a property shared with other proapoptotic drugs such as irufolven, a chemotherapeutic agent currently under clinical trials [27].

In reference to the mechanism of action of the depsipeptides, it has been observed that KF induces cell death preferentially via oncosis in tumor cells. KF-treated cells underwent a series of profound alterations including severe cytoplasmic swelling and vacuolization, dilatation and vesiculization of the endoplasmic reticulum, mitochondrial damage, and plasma membrane rupture, however, the nuclear envelope was preserved and no DNA degradation was detected [28]. Another depsipeptide, FR901228, is a histone deacetylase inhibitor and has been shown to acetylate histones H3 and H4 concomitant with induction of cell death by apoptosis in many solid tumors, T-cell leukemias and multiple myeloma [29]. FR901228 is currently under clinical trials for B-cell chronic lymphocytic leukemia treatment. Increased acetylation of H3 and H4 histones upon treatment with AT514 was not observed (data not shown) indicating that both depsipeptides activate different mechanisms for the induction of apoptosis. In a recent paper, we have been able to demonstrate that AT514 interferes with the Akt/NF-kB survival pathway, inducing Akt dephosphorylation at Ser 473 and decreasing NF-kB activity by dramatically reducing the levels of the p65 NF-kB component in B-cell chronic lymphocytic leukemia [30]. However, the molecular target of AT514 remains to be elucidated.

Differences were observed in the response of the two breast cancer cells, MCF-7 and MDA-MB-231, to AT514 treatment. MCF-7 cells underwent cell cycle arrest in G_0/G_1 while MDA-MB-231 died by apoptosis at the same doses and times. The latter is a cell line that has mutated p53, a protein able to induce cell cycle arrest or apoptosis depending upon the level of DNA damage. This suggested the possibility that accumulation of active p53 could be mediating the cell cycle blockade induced by AT514. However, since no p53 accumulation was observed in MCF-7 cells at the cytostatic conditions, this possibility could be ruled out. Moreover, at cytotoxic conditions, we could observe a p53 increase in both wild-type p53 cells used (MCF-7 and A2780 SC1 p53+). Therefore, cellular responses to AT514 exposure are triggered either in wild-type or mutated p53 cells. This suggests that p53 is not essential for the execution of these processes but p53 is implicated when it has a functional status. This property is a potential therapeutic advantage, as p53 is mutated in the vast majority of human tumors [31]. Another possible explanation for the delayed apoptosis in MCF-7 cells might be due to caspase 3 deficiency caused by a gene mutation [32]. This caspase is the most important effector caspase described, and deficiency in its gene product could confer a slight resistance to the drug. This phenomenon has already been characterized in these cells in response to other proapoptotic factors such as UV light [33]. The induction of apoptosis by AT514 even in the absence of caspase 3 is another promising therapeutic property.

Taken together, these results suggest that relatively low concentrations of AT514 induce cell cycle arrest and apoptosis in cancer cells irrespectively of p53 status raising the possibility of its use as a new anticancer drug.

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REFERENCES

[1] Ballard CE, Yu H, Wang B. Recent developments in depsipeptide research. Curr Med Chem 2002;9:471–98.
[2] Hochster H, Oratz R, Ettinger D, Borden E. A phase II study of Didemnin B (NSC 325319) in advanced malignant melanoma: an Eastern cooperative oncology group study (PB687). Invest New Drugs 1999;16:259–63.
[3] Brown AP, Morrissey RL, Faircloth GT, Levine BS. Preclinical toxicity studies of Kahalalide F, a new anticancer agent: single and multiple dosing regimens in the rat. Cancer Chemother Pharmacol 2002;50:333–40.
[4] Fecteau KA, Mei J, Wang HC. Differential modulation of signaling pathways and apoptosis of ras-transformed 10T1/2 cells by the depsipeptide FR901228. J Pharmacol Exp Ther 2002;300:890–9.
[5] Green D. Apoptotic pathways: the roads to ruin. Cell 1998;94:695–8.
[6] Cory S, Adams J. The Bcl2 family: regulators of the cellular life-or-death switch. Nat Rev Cancer 2002;2:647–56.
[7] Salvesen G, Dixit V. Caspases: intracellular signaling by proteolysis. Cell 1997;91:443–6.
[8] Kroemer G, Reed J. Mitochondrial control of cell death. Nat Med 2000;6:513–9.
[9] Los M, Burek CJ, Stroh C, Benedyk K, Hug H, Mackiewicz A. Anticancer drugs of tomorrow: apoptotic pathways as targets for drug design. Drug Discov Today 2003;8:67–77.
[10] Fisher D. Apoptosis in cancer therapy: crossing the threshold. Cell 1994;78:539–42.
[11] Matsuyama T, Bhasin A, Harshey R. Mutational analysis of glagellum-independent surface spreading of *Serratia marcescens* 274 on a low-agar medium. J Bacteriol 1995;177:987–91.
[12] Miyazaki Y, Oka S, Hara-Hotta H, Yano I. Stimulation and inhibition of polymorphonuclear leukocytes phagocytosis by lipoamino acids isolated from *Serratia marcescens*. FEMS Immunol Med Microbiol 1993;6:265–72.
[13] Hassal CH, Moschidis MC, Thomas WA. The conformations of serratamolide and related cyclotradepsipeptides in solution. J Chem Soc (B) 1971;1757–61.
[14] Branch P, Masson M, Aquilina G, Bignami M, Karran P. Spontaneous development of drug resistance: mismatch repair and p53 defects in resistance to cisplatin in human tumor cells. Oncogene 2000;28:3138–45.
[15] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Meth 1983;65:55–63.
[16] Kasibhatla S, Tseng B. Why target apoptosis in cancer treatment? Mol Cancer Ther 2003;2:573–80.

[17] Sarabia F, Chamma S, Ruiz AS, Ortiz LM, Herrera JL. Chemistry and biology of cyclic depsipeptides of medicinal and biological interest. Curr Med Chem 2004;11:1309–32.
[18] Byrd JC, Shinn C, Ravi R, Willis CR, Waselenko JK, Flinn IW, et al. Depsipeptide (FR901228): a novel therapeutic agent with selective, in vitro activity against human B-cell chronic lymphocytic leukemia cells. Blood 1999;94:1401–8.
[19] Klisovic D, Katz S, Effron D, Klisovic MI, Wickham J, Parthun MR, et al. Depsipeptide (FR901228) inhibits proliferation and induces apoptosis in primary and metastatic human uveal melanoma cell lines. Ophthalmol Vis Sci 2003;44:2390–8.
[20] Fujiwara H, Yamakuni T, Ueno M, Ishizuka M, Shinkawa T, Isobe T, et al. IC101 induces apoptosis by Akt dephosphorylation via an inhibition of heat shock protein 90-ATP binding activity accompanied by preventing the interaction with Akt in L1210 cells. J Pharmacol Exp Ther 2004;310:1288–95.
[21] Cory S, Adams JM. The Bcl2 family: regulators of the cellular life-or-death switch. Nat Rev Cancer 2002;2:647–56.
[22] Hou QH, Cymbalyuk SC, Hsu M, Hsu YT. Apoptosis modulatory activities of transiently expressed Bcl-2: roles in cytochrome c release and Bax regulation. Apoptosis 2003;8:617–29.
[23] Wu S, Zhu H, Gu J, Zhang L, Teraishi F, Davis JJ, et al. Induction of apoptosis and down-regulation of Bcl-X_L in cancer cells by a novel small molecule, 2[[3-(2,3-dichlorophenoxy)propyl]amino] ethanol. Cancer Res 2004;64:1110–3.
[24] Korsmeyer SJ, Wei MC, Saito M, Weiler S, Oh KJ, Schlesinger PH. Proapoptotic cascade activates BID, which oligomerizes BAK or BAX into pores that result in the release of cytochrome *c*. Cell Death Differ 2000;7:1166–73.
[25] Peart MJ, Tainton KM, Ruefli AA, Dear AE, Sedelies KA, O'Reilly LA, et al. Novel mechanisms of apoptosis induced by histone deacetylase inhibitors. Cancer Res 2003;63:4460–71.
[26] Cregan SP, Dawson VL, Slack RS. Role of AIF in caspase-dependent and caspase-independent cell death. Oncogene 2004;23:2785–96.
[27] Liang H, Salinas RA, Leal BZ, Kosakowska-Cholody T, Michejda CJ, Waters SJ, et al. Caspase-mediated apoptosis and caspase-independent cell death induced by irofulven in prostate cancer cells. Mol Cancer Ther 2004;3:1385–96.
[28] Suarez Y, Gonzalez L, Cuadrado A, Berciano M, Lafarga M, Muñoz A. Kahalalide F, a new marine-derived compound, induces oncosis in human prostate and breast cancer cells. Mol Cancer Ther 2003;2:863–72.
[29] Aron JL, Parthun MR, Marcucci G, Kitada S, Mone AP, Davis ME, et al. Depsipeptide FR901228 induces histone acetylation and inhibition of histone deacetylase in chronic lymphocytic leukemia cells concurrent with activation of caspase-8-mediated apoptosis and down-regulation of c-FLIP protein. Blood 2003;102:652–8.
[30] Escobar-Díaz E, López-Martín EM, Hernández del Cerro M, Puig-Kroger A, Soto-Cerrato V, Montaner B, et al. AT514, a cyclic depsipeptide from *Serratia marcescens*, induces apoptosis of B-chronic lymphocytic leukemia cells: interference with the Akt/NF-kappaB survival pathway. Leukemia 2005;19(4):572–9.
[31] Lowe SW, Ruley HE, Jacks T, Housman DE. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell 1993;74:957–67.
[32] Janicke RU, Sprengart ML, Wati MR, Porter AG. Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. J Biol Chem 1998;273:9357–60.
[33] Sasai K, Yajima H, Suzuki F. Suppression of postmitochondrial signaling and delayed response to UV-induced nuclear apoptosis in HeLa cells. Jpn J Cancer Res 2002;93:275–83.