약학석사학위논문

Marine compound palytoxin induces apoptotic cell death *via* Mcl-1 and Bcl-2 down-regulation in human leukemia cells

해양화학물 Palytoxin에 백혈병 세포사멸유도 및 Mcl-1과 Bcl-2 기능 억제 연구

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ABSTRACT

Palytoxin induces apoptotic cell death *via* Mcl-1 and Bcl-2 down−regulation in human leukemia cells

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Marine ecosystems contribute to a huge repository of pharmacologically active compounds. Palytoxin, one of the most toxic marine compounds, is known to be involved in the transformation of Na^+/K^+-ATPase into a cation channel inducing massive intracellular Na^+ influx. Anti-cancer activity of palytoxin is an emerging area of research and especially palytoxin-induced cancer cell death mechanisms remain to be elucidated. Here we show that palytoxin induced cell death of various leukemia cell lines at low picomolar concentrations. Importantly, palytoxin did not affect viability of peripheral blood mononuclear cells (PBMC) cells from healthy donors and did not create systemic toxicity in zebrafish, thus demonstrating excellent differential toxicity. Cell death was characterized by nuclear condensation as demonstrated by Hoechst staining as well as by caspase activation demonstrated by western blot and luminescent caspase assays. Palytoxin triggers cleavage of initiator pro-caspases-8 and -9 as well as executioner pro-caspases-3 and -7 after 6 hours of treatment in a dose-dependent manner. As caspase activation is sensitive to pan-caspase inhibitor zVAD, we conclude that palytoxin induces apoptotic cell death. From a molecular point of view, palytoxin down-regulates anti-apoptotic Bcl-2 family proteins Mcl-1 and Bcl-xL in a dose-dependent manner. MG-132, a proteasome inhibitor, was able to prevent

proteolysis of Mcl-1 whereas the three major proteasomal enzymatic activities were up-regulated by palytoxin. Palytoxin-induced dephosphorylation of Bcl-2 further exacerbates the pro-apoptotic effect of Mcl-1 and Bcl-xL degradation. As okadaic acid could rescue cell death triggered by palytoxin, we hypothesize involvement of protein phosphatase (PP)2A in Bcl-2 phosphorylation and induction of apoptosis by palytoxin. Altogether, we provide here first evidence of the role of palytoxin as a very potent and promising cancer-specific cytotoxic agent acting at low picomolar concentrations.

Keyword: Palytoxin; Cell death; Apoptosis; Mcl-1; Leukemia

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LIST OF ABBREVIAIONS

ATP	Adenosine triphosphate
Bcl-2	B-cell leukemia/lymphoma 2
Bcl-xL	B-cell lymphoma extra like large
BID	Bcl-2 interacting domain death agonist
BIM	Bcl-2 interacting mediator of cell death
BSA	Bovine serum albumin
BH-3	Bcl-2 Homology domain 3
β-TrCP	β-transducin repeat containing protein 1
Ca^{2+}	Calcium ions
Caspase	Cysteine aspartate cleaving protein
DMSO	Dimethylsulfoxide
DPBS	Dulbecco's phosphate buffered saline
ERK1/2	Extracellular signal regulated kinase 1/2
FBW7	F-box and WD repeat domain containing 7
IC_{50}	half maximal inhibitory concentration
IL-8	Interleukin 8
IκB-α	Inhibitor of kappa alpha
Jurkat	Human T-cell lymphoma cell line
K562	Human chronic myelogenous leukemia cell line
MAPK	Mitogen-activated protein kinase
Mcl-1	Myeloid cell leukemia 1
MPER	Mammalian Protein Extraction Reagent
Mule	Mcl-1 ubiquitin ligase E3

Na^+/K^+-ATPase	Sodium-potassium adenosine triphosphatase
NOXA	Phorbol-12-myristate-13-acetate-induced protein 1
NF-κB	Nuclear factor-kappa B
PUMA	p53 up-regulated modulator of apoptosis
Ok	Okadaic Acid
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with Tween
PBMC	*Peripheral blood mononuclear cell*
pBcl-2	Phospho B-cell lymphoma 2 serine 70 residue
PI	Propidium Iodide
PTU	phenylthiourea
PMSF	Phenylmethylsulphonyl fluoride
PP2A	Protein phosphatase 2A
PVDF	Polyvinylidene fluoride
p38	p38 mitogen activated protein kinase
RPMI	Roswell Park Memorial Institute
SCF	Skp Cullin F-box containing
SDS	Sodium dodecyl sulfate
SDS-Page	SDS-Polyacrylamide Gel Electrophoresis
U937	Human acute myelogenous leukemia cell line

INTRODUCTION

Palytoxin is originally found in soft corals from tropical areas of the Pacific Ocean, for example from Hawaii where it was discovered in the tropical soft coral *Palythoa* species, a zoanthid [1]. Palytoxin was first characterized by the chemist Moore in 1981. This compound represents one of the largest polyether-type phycotoxins [1]. This non-peptidic toxin consists of a long polyhydroxylated and partially unsaturated chain of 129 carbon atoms and the aliphatic backbone contains 64 chiral centers [2]. Eight double bonds are able to exhibit cis/trans isomerism so that palytoxin could present over 10^{21} stereoisomers [3]. Structural analogs of palytoxin were also discovered and include ostreocin [4], ovatoxin [5], and mascarenotoxin [6]. The molecular weight of palytoxin ranges from 2658 to 2680 Da, depending on the *Palythoa* species from which it was obtained [2]. Palytoxin features both lipophilic and hydrophilic regions and is referred to as a super-carbon-chain compound, since it has the longest chain of continuous carbon atoms in any known natural product [7]. It is heat-stable, not inactivated by boiling and is stable in neutral aqueous solutions for prolonged periods, however a rapid decomposition occurs under acid or alkaline conditions leading to loss of its toxicity [2].

Molecular action of palytoxin causes blockage of the Sodium-potassium adenosine triphosphatase (Na^+/K^+-ATPase) channel [8, 9]. This transmembrane protein exchanges three Na^+ ions and two K^+ ions using Adenosine triphosphate (ATP) hydrolysis as the driving force. The electrochemical gradient generated by the sodium pump for the maintenance of cell homeostasis is then inhibited by palytoxin. Palytoxin binds to the extracellular part of the Na^+/K^+-ATPase to transform the pump into a non-specific, permanently open ion channel causing membrane depolarization with massive increase of calcium ions (Ca^{2+}) into the cytosol [10] strongly interfering with some vital functions. An alteration of intracellular cation concentration, in particular a calcium increase, is generally associated with cell death

as a modification in the distribution of these ions across the plasma membrane plays a key role in palytoxin-induced cell-type specific cytotoxic response [11].

Further characterization of the mode of action of this compound would contribute to a better understanding of the molecular mechanism of cellular effects induced by palytoxin, which can be acting as a tool to probe the role of different types of signaling mechanisms involved in carcinogenesis. It has been already demonstrated to act as a skin tumor promoter able to modulate key signal transduction pathways involved in carcinogenesis [12]. In particular, it has been shown that palytoxin stimulates prostaglandin production from arachidonic acid and activates mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 mitogen activated protein kinase (p38) [13]. MAPKs mediate intracellular signaling associated with a variety of cellular activities such as proliferation, differentiation, survival, death and transformation [14]. Mitogenic agents, inflammatory cytokines or oxidative stress activate ERK and JNK signaling pathways. Subsequently, downstream MAPKs phosphorylate various protein substrates to eventually activate transcription factors that modulate inflammatory gene expression [15].

For our project, we focused our attention on the identification of cellular pathways and mechanisms involved in palytoxin-induced cell death in human leukemia cell lines. Intracellular signaling cascades induced by palytoxin may lead to activation of intrinsic and extrinsic apoptotic pathways triggered by alterations of the expression levels of pro- and anti-apoptotic Bcl-2 family proteins. Altogether our results show that palytoxin acts as a potent and promising cancer-specific cytotoxic agent at low picomolar concentrations.

MATERIALS AND METHODS

1. Cells and medium

K562 (human chronic myelogenous leukemia, DSMZ, Germany), Jurkat (human T-lymphocyte, DSMZ, Germany) and U937 (human histiocytic lymphoma, DSMZ, Germany) cells were cultured in Roswell Institute Park Memorial Institute (RPMI) 1640 medium (Bio-Whittaker, Lonza) containing 1% (v/v) with a antibiotic/antimycotic mixture of penicillin 100U/ml, streptomycin 100µg/ml, amphotericin B 0.25µg/ml (Bio-Whittaker, Lonza) at 37°C and 5% of CO_2 in a humidified atmosphere. Cells were cultured and harvested every three days and treated with palytoxin in exponential growth phase. Cells were regularly tested against mycoplasma infection (Mycoalert™, Lonza USA) according to the manufacturer's instructions.

2. Compounds

Palytoxin was a gift of Coral Biome, Marseille, France with a molecular mass of 2,680.14 g mol^{-1}. Compound was received as a powder and solubilized in DMSO (Sigma-Aldrich, USA) and further diluted to get working aliquots at 1mM. Stocks and aliquots were stored at 4°C and protected against light and were used directly prior to the experiments. Etoposide was purchased from (Sigma-Aldrich, USA), dissolved in DMSO at a stock concentration of 50mM. Pan-caspase inhibitor, z-VAD FMK was purchased from Calbiochem (San Diego, USA) dissolved in DMSO and added 1h before at the concentration of 50µM. Protein phosphatase 2A inhibitor okadaic acid was purchased from Calbiochem (San Diego, USA), dissolved in DMSO at a stock concentration of 1mM. Proteasome inhibitor MG-132 was purchased from (Sigma-Aldrich, USA) dissolved in DMSO at a stock concentration of 10mM. MAP kinase inhibitors, SB202190 and PD98059 were purchased from Calbiochem (San Diego, CA, USA), dissolved in DMSO at a stock concentration of 100mM. Hydroquinone was purchased from (Sigma-Aldrich, USA), dissolved in DMSO at 10mM. Inhibitors were used 1h before palytoxin treatment, at indicated working concentrations (Table I).

Table I: Compounds used with corresponding working concentrations.

Name	Molecular mechanism	Concentration	Supplier
Etoposide	Caspase activator	100 μ M, 4h	Sigma Aldrich
z-VAD FMK	Pan-Caspase inhibitor	10 μ m, 1h	Calbiochem
MG-132	Proteasome inhibitor	5 μ M, 1h	Sigma Aldrich
Paclitaxel	Bcl-2 phosphorylation	500nM, 8h	Sigma Aldrich
SB202190	p38 inhibitor	10 μ M, 1h	Calbiochem
PD98059	ERK 1/2 inhibitor	10 μ M, 1h	Calbiochem
Okadaic Acid	PP2A inhibitor	10 μ M, 1h	Calbiochem
Hydroquinone	PP2A activator	10 μ M, 24h	Sigma Aldrich

3. Cell viability assessment

Cell vitality was assessed in three different leukemia cell lines K562, Jurkat and U937. Each cell line was treated with different concentrations of palytoxin for 2, 4, 6, 8 hours in a dose and time-dependent manner. Percentage of viability was evaluated using Trypan Blue exclusion test (0.2% Trypan Blue) and expressed as percentage of viability of cells without palytoxin treatment considered to be 100%.

4. Caspase 3/7 activity assay

Three x 10^5 U937 cells were seeded in 96-well plates in triplicate in 75µl for palytoxin treatment. Enzymatic activities of caspases- 3 and -7 were determined by using a luminescent Caspase-glo®3/7 Assay) (Promega, South Korea). The assay was performed according the manufacturer's instruction by adding 75µl of caspase-3/7 substrate to U937 cells in suspension. After one hour of incubation with DEVD-aminoluciferin substrate and luciferase, the luminescent signal was measured using an Centro LB 960 Microplate Luminometer (Berthold, South Korea).

5. Proteasome activity assay

The Proteasome-Glo® cell based assay (Promega, South Korea) was used to evaluate the three major proteolytic enzyme activities (chymotrypsin-like, caspase-like, trypsin-like). The assays were performed as indicated in the manufacturer's protocol. U937 cells, at a concentration of 10^6 cells/ml in RPMI 1640 medium containing 0.1% FCS, were treated at indicated concentrations of palytoxin. After an incubation period, 50µl of U937 cellular suspension were mixed with 50µl of assay reagent. The luminescence signal was measured on Centro LB 960 Microplate Luminometer (Berthold, South Korea). Proteasome signal was normalized to the number of viable cells performed in parallel. MG-132 at 5µM (Sigma, USA) was used as a control for proteasomal inhibition.

6. Cell lysate preparations and western blots

Ten millions of U937 cells were seeded in 30ml in RPMI 1640 (Lonza, USA) with 10% foetal bovine serum (FBS) (Biowest, USA), 1% antibiotics (Lonza, USA). Cells were treated with palytoxin in a dose- and time-dependent manner as indicated. Cellular lysates were centrifuged at 22°C, 350g for 7 minutes. After removal of the supernatant, pellets were washed in 1ml phosphate-buffered saline (PBS) and centrifuged again at 4°C, 350g for 7 minutes. Afterwards, supernatants were removed and pellets were directly stored at -80°C until use. The extraction was performed on ice to avoid denaturation of proteins. Whole-cell extracts were prepared with 5ml of mammalian protein Extraction reagent (M-PER), according to the manufacturer's instructions (Pierce, Rockford USA). Extraction reagents include protease inhibitor 40µl/ml (Sigma, USA), phenylmethylsulphonyl fluoride (PMSF) 1mM (Roche, USA), sodium orthovandate 1mM (Sigma, USA), Phosphostop inhibitor 100µl/ml (Roche, USA) and M-PER in a final volume of 5ml. Depending on the pellet size, 500µl of reagents were used for control pellets and 300µl for treated cell pellets. Dissolved pellets were centrifuged for 25min, 4°C, 18,000g speed. Aliquots were stored at -80°C.

Proteins from total extracts were separated by size using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-Page) and transferred onto a Polyvinylidene fluoride (PVDF) membrane (GE healthcare) and blocked with 5% non-fat milk or 5% bovine serum albumin (BSA) in PBS-Tween overnight. Equal loading of samples was controlled by using β-actin. Blots were incubated with primary antibodies: anti β-actin (1/10000, Sigma-Aldrich A5441), anti Caspase-3 (1/1000, Santa Cruz 56053), anti Caspase-7 (1/1000, Cell Signaling 9494) anti Caspase-8 (1/1000, Cell Signaling 9746), anti Caspase-9 (1/1000, Cell Signaling 9502), anti Mcl-1 (1/1000, Cell Signaling 4572), anti BID (1/1000, Cell Signaling 2002), anti pBcl-2 ser70 (1/2000, Cell signaling 2827), anti Bcl-2 (1/2000, Calbiochem OP60), anti Bcl-xL (1/1000, BD biosciences 610212). All antibodies were diluted in a PBS-Tween solution containing 5% BSA or 5% non-fat milk according to the provider's protocols. After incubation with primary antibodies, membranes were washed 3 x 10 min with PBS-Tween, followed by an incubation of 1h at RT with the corresponding secondary (HRP-conjugated) antibodies. After washing 3 x 10 min with PBS-Tween, specific immunoreactive proteins were visualized by autoradiography using the ECL Plus Western Blotting Detection System Kit® (GE Healthcare, South Korea).

Table II: Antibodies used and working conditions

Target Protein	Supplier	Origin Animal	Primary Antibody	Secondary Antibody
β-Actin	Sigma Aldrich # A5441	Mouse	1/10,000 PBS-T 5% milk Room Temperature 1h.	1/4,000 PBS-T 5% milk Room Temperature 1h
Caspase 3	Santa Cruz SC 56053	Mouse	1/1000 PBS-T 5% milk overnight 4°C.	1/4,000 PBS-T 5% milk Room Temperature 1h
Caspase 7	Cell Signaling # 9494	Mouse	1/1000 PBS-T 5% milk overnight 4°C.	1/4,000 PBS-T 5% milk Room Temperature 1h
Caspase 8	Cell Signaling #9746	Mouse	1/1000 PBS-T 5% BSA overnight 4°C.	1/4,000 PBS-T 5% milk Room Temperature 1h
Caspase 9	Cell Signaling #9502	Rabbit	1/1000 PBS-T 5% milk overnight 4°C.	1/5,000 PBS-T 5% milk Room Temperature 1h
Mcl-1	Cell Signaling #4572S	Rabbit	1/1000 PBS-T 5% milk overnight 4°C.	1/4,000 PBS-T 5% milk Room Temperature 1h
Bcl-xL	BDBiosciences#610212	Rabbit	1/1000 PBS-T 5% milk overnight 4°C.	1/4,000 PBS-T 5% milk Room Temperature 1h
Bcl-2	Calbiochem # OP60	Mouse	1/1000 PBS-T 5% milk overnight 4°C.	1/5,000 PBS-T 5% milk Room Temperature 1h
BID	Cell Signaling # 2002	Rabbit	1/1000 PBS-T 5% milk overnight 4°C.	1/5,000 PBS-T 5% milk Room Temperature 1h
pBcl-2 ser70	Cell Signaling #2827	Rabbit	1/2000 PBs-T 5% BSA overnight 4°C.	1/5,000 PBS-T 5% milk Room Temperature 1h

7. Fluorescent microscopy analysis

Three x 10^5 U937 cells were seeded in 24-well plates prior to palytoxin treatment. 300µl of treated cells were transferred into another 24-well plate for double staining with (1) the DNA-specific dye Hoechst 33342 (Sigma, USA) at 1µl/mg for 30 minutes of incubation and (2) propidium iodide (Sigma, USA) at 1.5µl/mg, diluted in PBS for 15 minutes of incubation. Induction of apoptosis was assessed by fluorescent microscopy (Nikon TI-U, Seoul Korea) and expressed as the percentage of cells presenting condensed nuclei. Different stages of nuclear fragmentation were considered and at least 300 cells were counted in three independent fields. Microscopy images were analyzed using the ImageJ software (http://rsb.info.nih.gov/ij/docs/index.html).

8. Systemic toxicity in zebrafish

Zebrafish (*Danio rerio*) were obtained from the "Zebrafish International Resource Center" (ZIRC, OR) and maintained according to the zebrafish guidelines [16]. Adult fish were kept at 28.5°C on a 14 hour light/10 hour dark cycle similar to natural conditions and all embryos collected from natural mating as a unit of hours-post fertilization (hpf). 0.003% phenylthiourea (PTU) was added 14 hour before palytoxin treatment in order to remove pigmentation of zebrafish models. Embryos were then treated with indicated concentration of palytoxin in 24 hpf. Photographs were taken under light microscopy (Carl Zeiss Stereo microscope DV4, Seoul Korea) in order to quantify viable zebrafish. We thank Prof. Kyu-Won Kim for the generous help afforded during the set-up and execution of these experiments.

9. Differential toxicity effects on healthy peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were purified from fresh buffy coats of four healthy adult human donors (Red Cross, Luxembourg) using the standard Ficoll-Hypaque (GE Healthcare, Roosendaal, The Netherlands) density separation method. All healthy volunteer donors gave informed consent. After three washes in Dulbecco's Phosphate Buffered Saline (DPBS, Lonza), cells were counted, and then re-suspended in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum and 1% antibiotics-antimycotic at a cell density of 2 x 10^6 cells/ml. Cells were treated with palytoxin at indicated concentrations. After 8 hours of incubation, Trypan Blue assay was used to analyze PBMC cell viability.

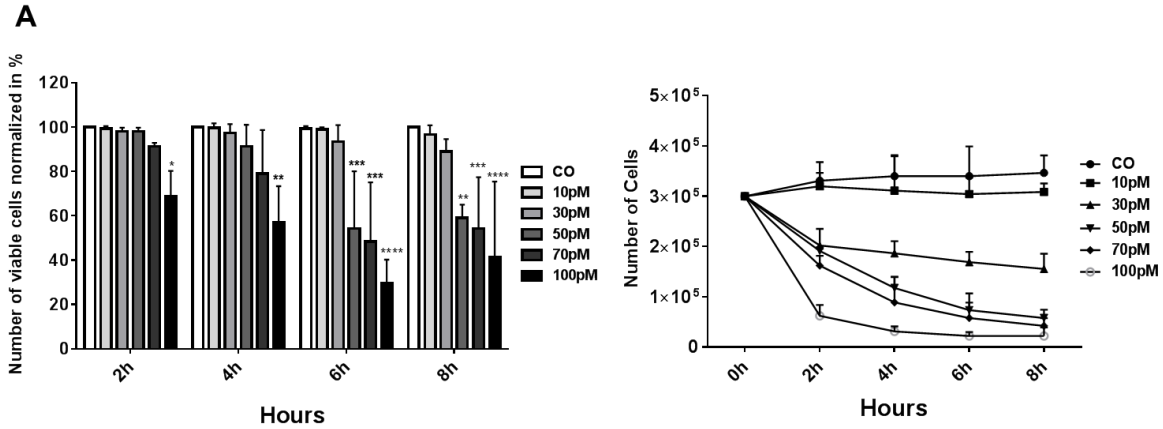
10. Statistical Analysis

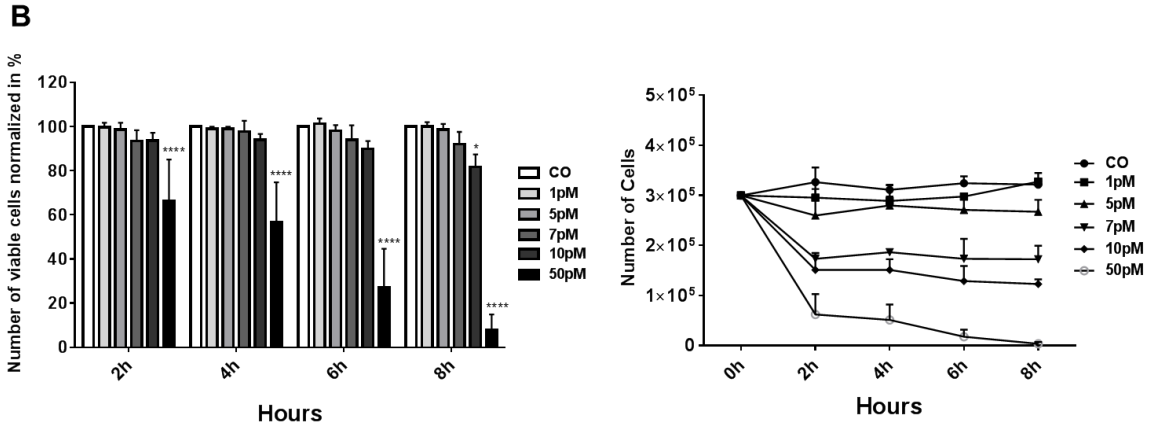
Data are presented as mean of at least three independent experiments with standard deviations. Statistical analysis was performed using the GraphPad Prism 6.0 software. One-way and two-way ANOVA followed by Holm-Sidak multiple comparison tests were used for statistical comparisons. P-values below 0.05 were considered as statistically significant.

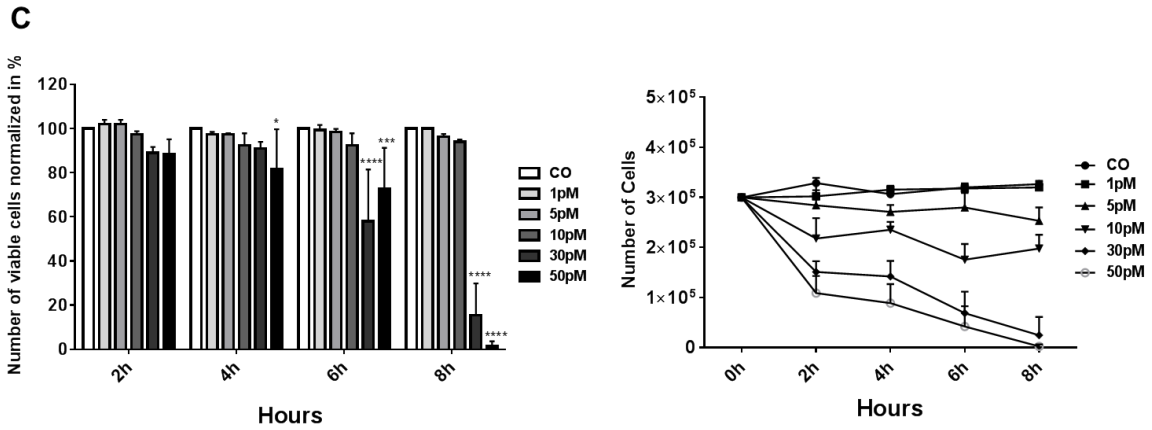
RESULTS

1. Cytotoxic effect of palytoxin on human leukemia cells

In order to assess the effect of palytoxin on the induction of cell death mechanisms in human leukemia cell lines, we first evaluated the effect of palytoxin on the cell growth of chronic leukemia (K562), acute T-cell leukemia (Jurkat), and histiocytic lymphoma (U937) cell lines (**Figure 1.A, 1.B, 1.C**). Leukemia cells were treated for 2, 4, 6, and 8h at different concentrations of palytoxin and viability was assessed by Trypan Blue exclusion assay. **Figure 1.D** shows that palytoxin strikingly inhibits leukemia cell growth in a dose-dependent manner at low picomolar concentrations. Among our leukemia cell models, K562 cells appeared to be more resistant to the palytoxin treatment (IC_{50}: 52.1 ± 5.9pM) in comparison to Jurkat (IC_{50} 8.7 ± 1.0pM) and U937 cells (IC_{50}: 9.0 ± 0.7pM).







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K562	IC_{50} (pM)	Jurkat	IC_{50} (pM)	U937	IC_{50} (pM)
2h	52.1 ± 5.9	2h	12.8 ± 6.0	2h	24.8 ± 1.7
4h	32.8 ± 7.8	4h	13.5 ± 2.1	4h	27.3 ± 8.1
6h	28.5 ± 7.3	6h	8.8 ± 1.0	6h	14.0 ± 6.1
8h	24.9 ± 5.0	8h	8.7 ± 1.0	8h	9.0 ± 0.7

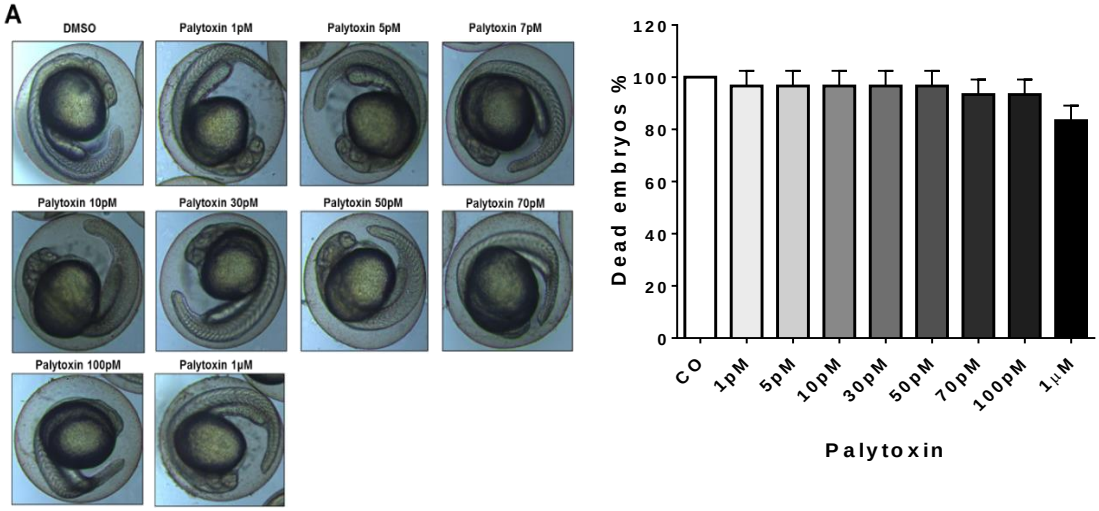
Trypan Blue assay – Calculated IC50 values (pM)

Figure 1. Cytotoxic effect of Palytoxin on human leukemia cell lines

(A) Effects of Palytoxin on human chronic leukemia K562 cell viability and proliferation (B) Effects of Palytoxin on human T-cell leukemia Jurkat cell viability and proliferation (C) Effects of Palytoxin on human histiocytic lymphoma U937 cell lines. (D) Calculated IC_{50} values of three different human leukemia cell lines. Data are the mean of SD ± of three independent cultures. *p < 0.05, **p < 0.01, ***p <0.005, ****p <0.001 versus control.

2. Effect of palytoxin on healthy cells and organisms

To evaluate the differential toxicity of palytoxin, we used both zebrafish and PBMCs from healthy donors **(Figure 2.A, 2.B)**. Palytoxin was used a various concentrations to treat zebrafish embryos and larvae for 24h. Interestingly, we did not observe any toxicity nor alterations of the morphology of the yolk-sac or axial/tail malformations (**Figure 2.A**). Moreover we treated PBMCs from healthy donors with palytoxin at various concentrations and conducted Trypan Blue staining to assess cell integrity and viability (**Figure 2.B**). Even at tumor-promoting concentrations, palytoxin did not generate levels of toxicity comparable to leukemia models. Altogether this compound demonstrates a remarkable differential toxicity and selectively targets cancer cells.



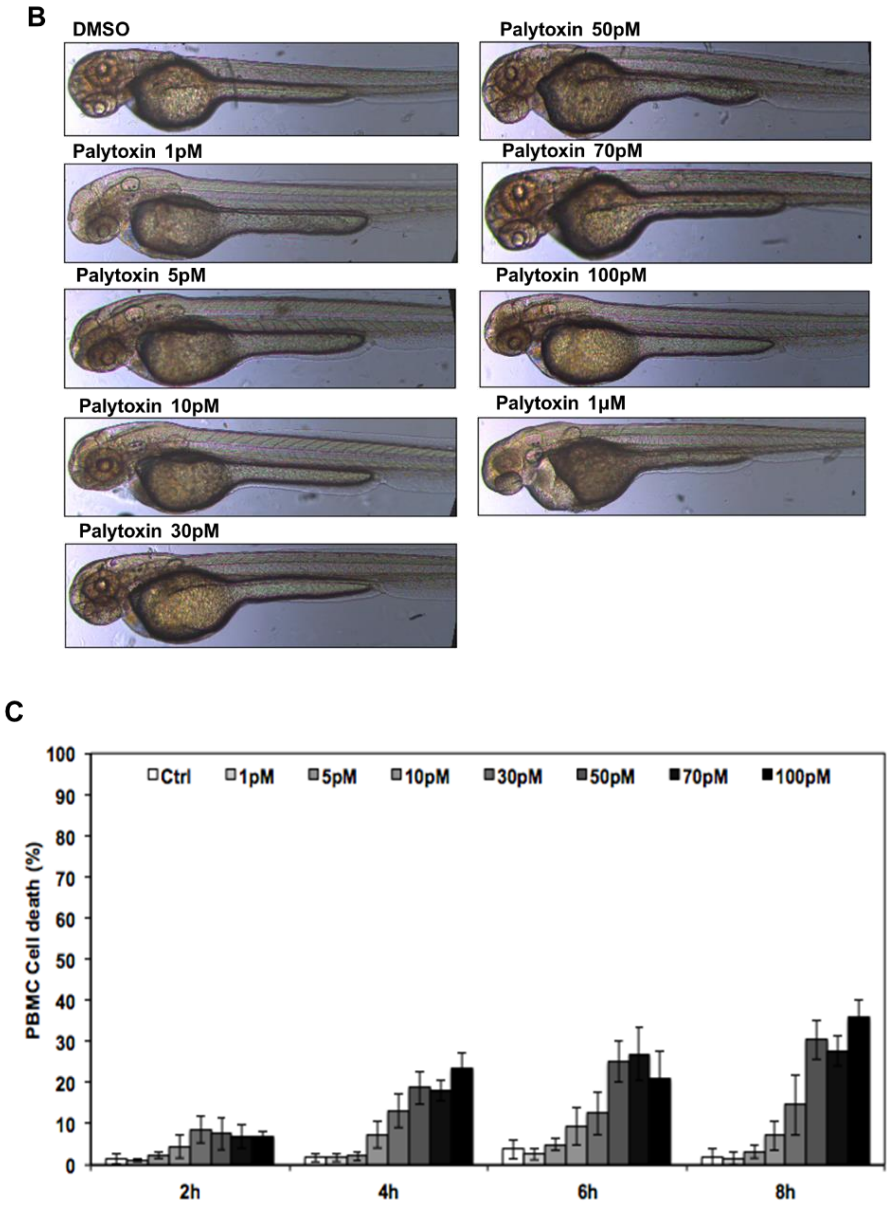


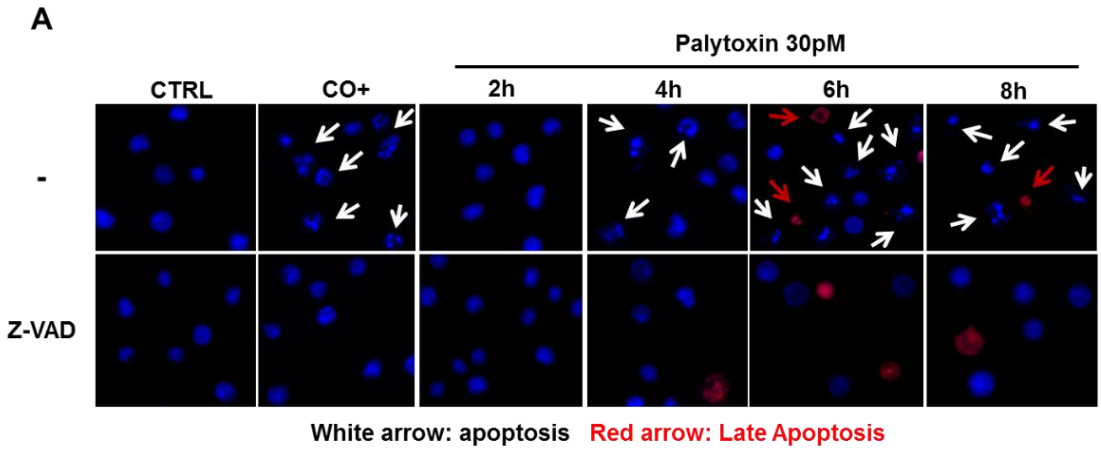
Figure 2. Effects of Palytoxin on zebrafish and healthy cells

(A) Effects of palytoxin on developmental defects in zebrafish embryos. Zebrafish embryos were incubated with palytoxin at different concentrations from 1pM-100μM for 24h. Bar graph showing the rate of dead embryos in palytoxin-treated at 24 hpf (*n*=10 fish examined). (B) Effect of palytoxin exposure on zebrafish morphology scale bar: 20μM. Zebrafish are exposed to palytoxin did not exhibit moderate or severe defects of morphology. (C) Effect of palytoxin on the viability of healthy PBMCs. PBMCs from healthy donors were treated at indicated palytoxin concentrations for 24h. Results

correspond to the mean of ± SD of the quantification of three independent experiments.

3. Palytoxin-induced cell death leads to caspase activation in U937 cells

We subsequently investigated whether palytoxin was able to trigger caspase-dependent apoptotic cell death. Palytoxin-induced cell death was identified by changes in the nuclear morphology typical of apoptotic cell death, as demonstrated by Hoechst and PI staining. (**Figure. 3A**) As caspase activation is sensitive to pan-caspase inhibitor zVAD, we concluded that palytoxin induces apoptosis (**Figure. 3B**). Palytoxin induced the cleavage of the pro-caspases-8 and -9 and executor pro-caspases-3 and -7 after 6h of treatment in a dose-dependent manner (**Figure. 3C**). These results were further confirmed by luminescent assays where palytoxin induced enzymatic activity of caspase-3 and 7 in U937 cells (**Figure. 3C**).



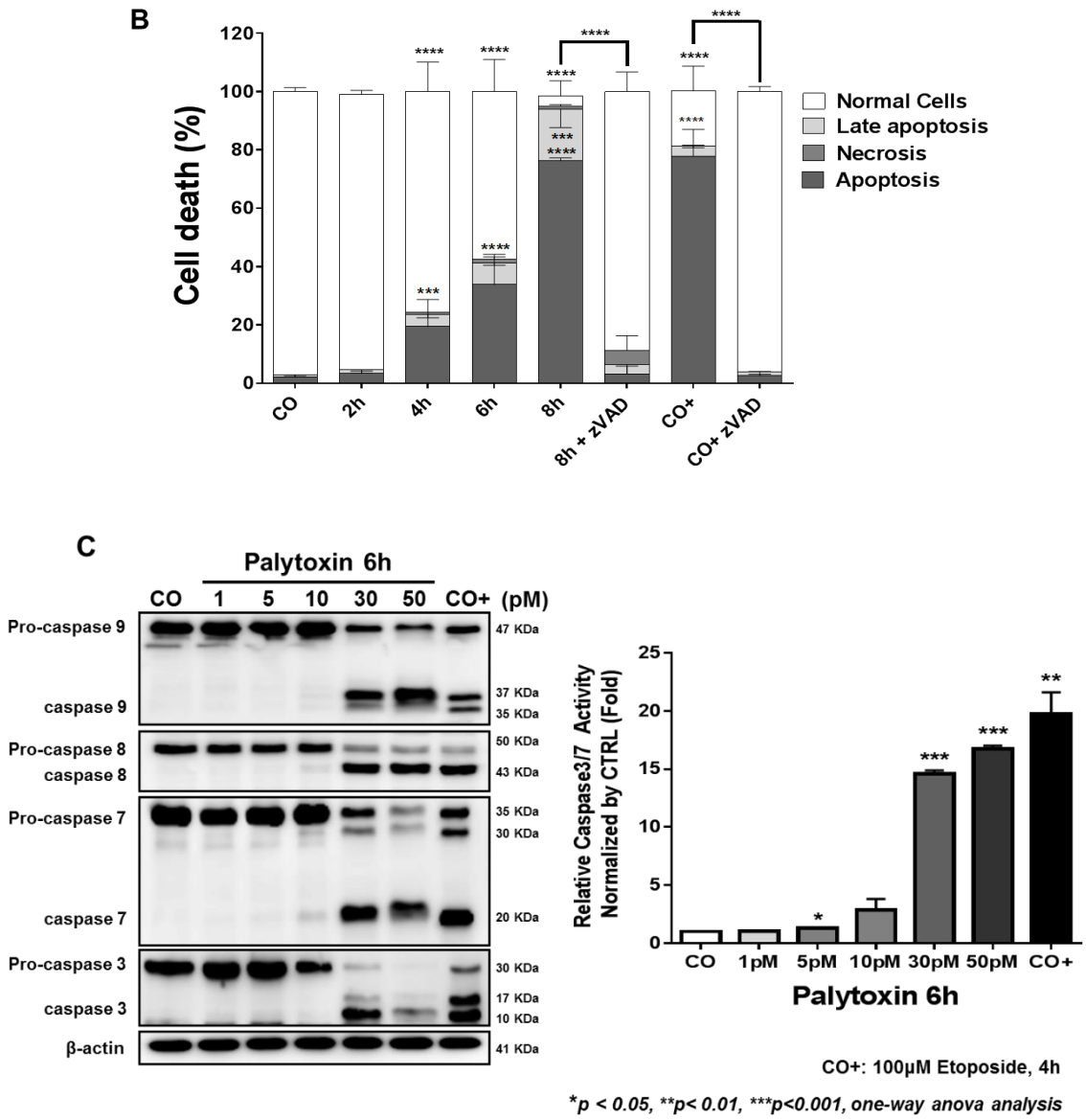


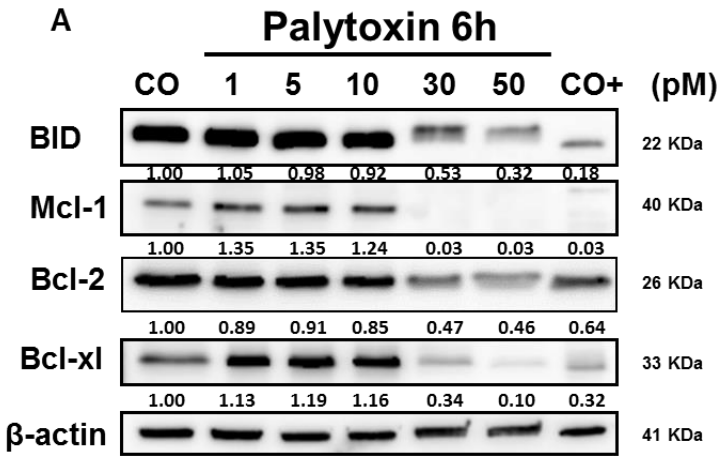
Figure 3. Palytoxin-induced cell death leads to caspase activation in U937 cells

(A) Hoechst/PI staining of palytoxin-treated cells. White arrows indicate cells displaying apoptotic features such as fragmented and condensed nuclei. Red arrows indicate PI positive cells undergoing late apoptosis and presenting necrotic morphology. (B) The percentage of apoptotic cells was evaluated by counting cells corresponding to apoptosis, late apoptosis, and necrosis. At least 100 cells in three random fields were counted by fluorescence microscopy. (C) Palytoxin activation of pro-caspasese-8, -9, -7, and -3 was investigated by western blot analysis (left; one of three independent experiments). Enzymatic activity of

caspases-3 and -7 were up-regulated in a dose-dependent manner. (right; data representative of three independent experiments (mean ± SD). Etoposide-treated cells (100μM, 4h) served as positive control for induction of apoptosis. *p < 0.05, **p < 0.01, ***p <0.005 versus control.

4. Palytoxin down-regulates expression of anti-apoptotic Bcl-2 family proteins

As the induction of apoptosis can be triggered by both inhibition of anti-apoptotic proteins or activation of pro-apoptotic mechanisms, we investigated the overall expression levels of selected proteins after palytoxin treatment (**Figure. 4A**). Expression level of myeloid cell leukemia-1 (Mcl-1) and B-cell lymphoma extra like large (Bcl-xL) are down-regulated in a dose-dependent manner whereas B-cell lymphoma 2 (Bcl-2) is affected to a lesser extend. Moreover, pro-apoptotic Bcl-2 interacting domain death agonist (BID) was truncated by palytoxin treatment (**Figure. 4B**), which implied that the amplification of intrinsic and extrinsic cell death pathway could be expected [17].



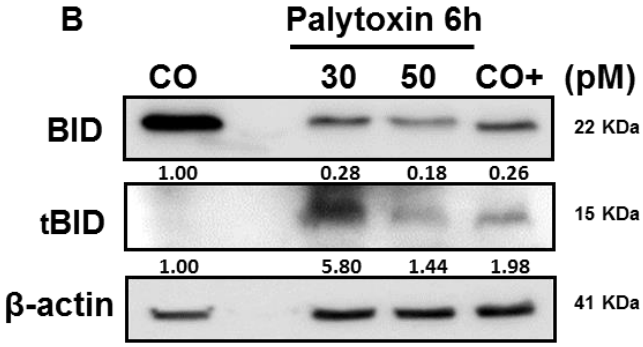


Figure 4. Palytoxin down-regulates expression of anti-apoptotic Bcl-2 family proteins

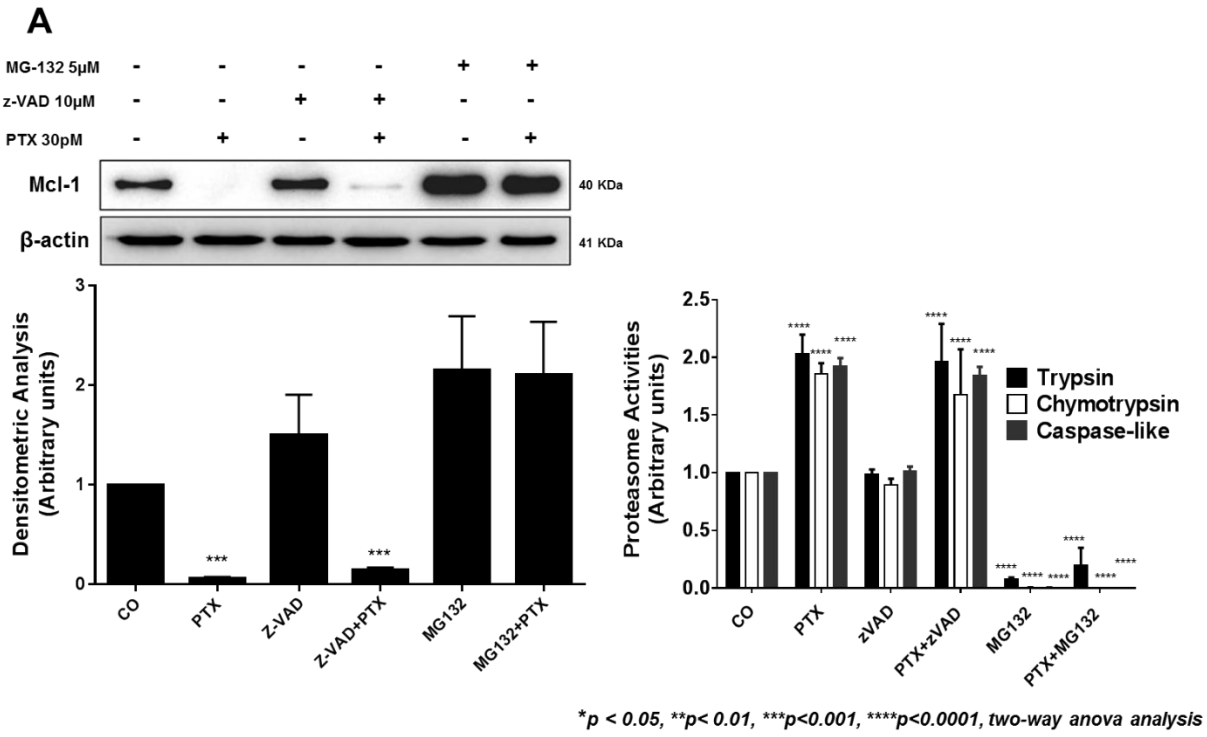
(A) Western blot analysis of anti-apoptotic Bcl-2 family protein expression inhibited by palytoxin after 6 hours of treatment. (B) Western blot analysis of the truncation of pro-apoptotic protein BID induced by palytoxin. Western blot results are representative of three-independent experiments.

5. Mcl-1 is down-regulated by palytoxin in a proteasome dependent manner

It is well established that anti-apoptotic Mcl-1 can be down-regulated by several mechanisms [18] including caspase-mediated- or proteasome-dependent degradation [19]. Accordingly, we assessed palytoxin-induced Mcl-1 degradation in the presence of two prototypical inhibitors, zVAD and MG-132. Our results show that palytoxin-induced Mcl-1 degradation is completely abrogated in the presence of MG-132 underlining the essential role of proteasome degradation in the effect of palytoxin (**Figure. 5A**). Unexpectedly, the three major proteasomal enzymatic activities including trypsin, chymotrypsin and caspase-like were up-regulated after palytoxin treatment most likely further exacerbating the degradation of Mcl-1.

In order to get deeper insight into the chronology of the molecular events triggered by palytoxin, we attempted to identify drivers of cell death rather than investigate the consequence of ongoing cellular demise. We used a kinetic approach to compare

proteasomal activation, Mcl-1 degradation and caspase activation. We obtained a rapid and significant activation of proteasomal activities prior to Mcl-1 degradation and caspase cleavage (**Figure 5.B**). In parallel, ATP levels were quantified in the cells and we also observed a rapid decline in ATP levels. (**Figure 5.B**). BID was cleaved at early steps which is in line with our hypothesis as BID requires polyubiquitination and proteasomal activation for its processing. Altogether, we conclude that palytoxin selectively targets essential cell death regulators prior to executioner caspase 3 activation and overall cellular degradation.



*p < 0.05, **p< 0.01, ***p<0.001, ****p<0.0001, two-way anova analysis

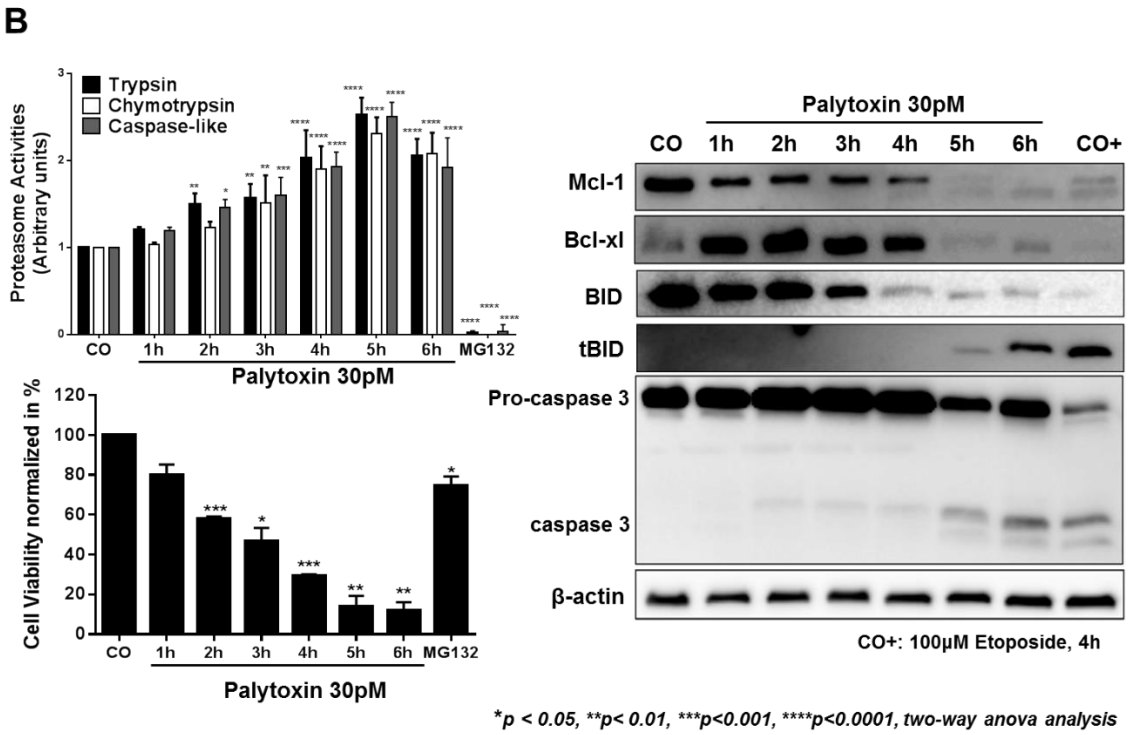


Figure 5. Mcl-1 is ubiquitously down-regulated by palytoxin in a proteasome dependent manner

(A) Palytoxin induced proteasome-dependent degradation of Mcl-1 in U937 cells. Band intensities were quantified using ImageJ software. Values represent the mean of ± SD for three independent experiments. The three major proteolytic activities of the 26S proteasome were assessed under the same conditions as the western blots. MG-132 at 5μM was used as a positive control for proteasomal inhibition in U937 cells. Asterisk indicates significant differences of treated versus control ***P< 0.005, ****P<0.001.

(B) Kinetic analysis of assessment of three major proteasome activities in U937 cells. ATP levels were determined in order to normalize the three major proteasome activities induced by palytoxin. Expression of Mcl-1 degradation was evaluated by western blot analysis under the same conditions. The experiments are representative of three independent experiments. *P<0.05, **P<0.01, ***P<0.005, ****P<0.001.

6. Bcl-2 serine 70 dephosphorylation induced by palytoxin is mediated through Protein Phosphatase 2A activation in U937 cells

Our results also show that palytoxin affects Bcl-2 expression and phosphorylation levels witnessed by a discrete upshift of the band [20] (**Figure. 6A**). We investigated phosphorylation levels of Bcl-2 as it is well known that serine 70 phosphorylation is required for the pro-survival function of this protein [21]. Our results show a dose-dependent dephosphorylation of Bcl-2 contributing potentially to a loss of its pro-survival function (**Figure. 6A**). Serine 70 phosphorylation of Bcl-2 can be abrogated by phosphatase activation, mainly by protein phosphatase 2A (PP2A) activity [22]. Accordingly, in a next step, we used chemical inhibitors of selected cell signaling pathways including okadaic acid used at a concentration inhibiting specifically PP2A. Our results show that PP2A inhibition partially rescues U937 cells from palytoxin-induced cell death whereas other kinase pathways do not seem to be involved. (**Figure. 6B**)

Altogether, we hypothesize that palytoxin-induced dephosphorylation of Bcl-2 by PP2A further exacerbates the pro-apoptotic effect of Mcl-1 and Bcl-xL degradation and that okadaic acid could rescue at least in part of cell death trigged by palytoxin.

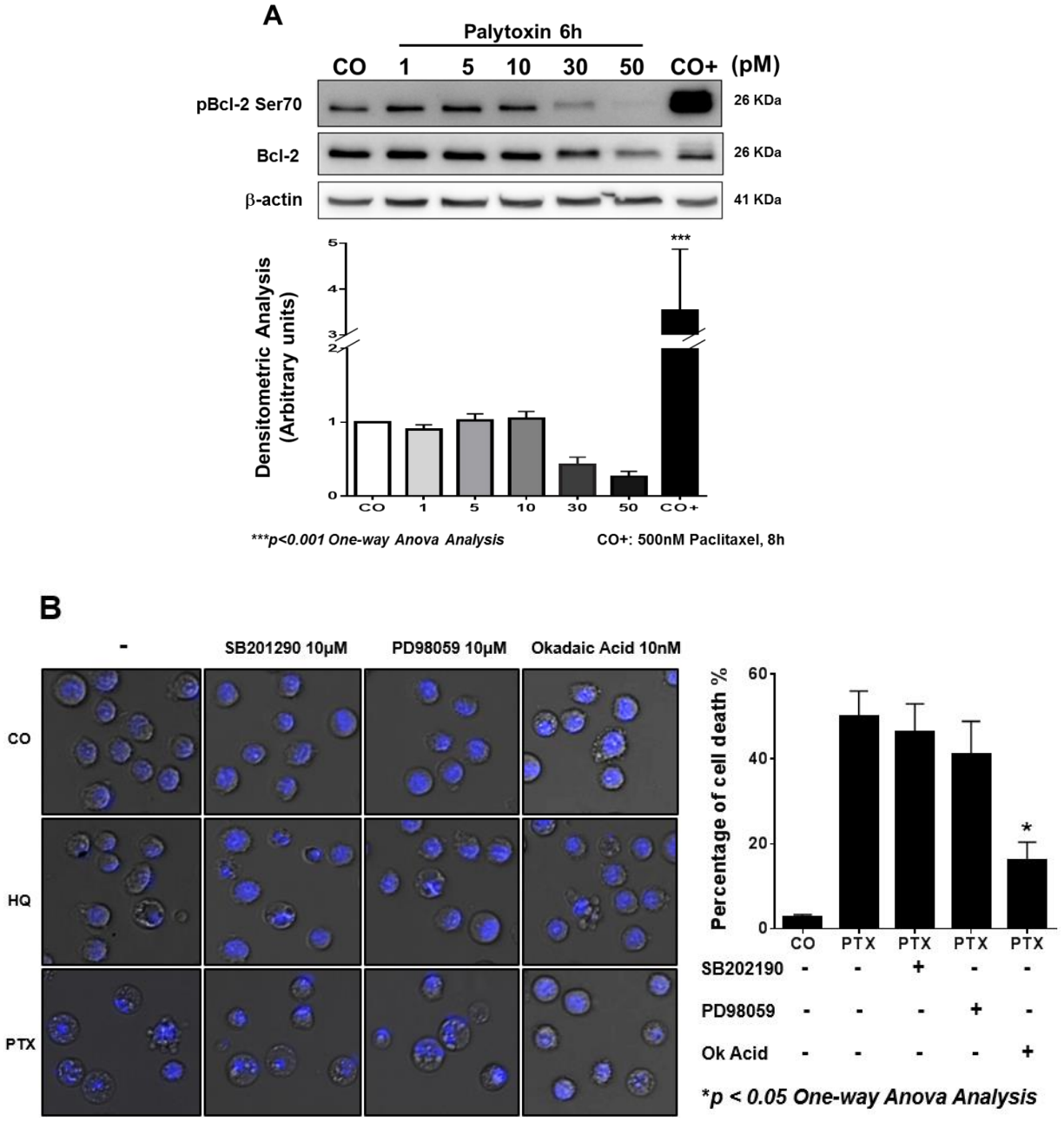


Figure 6. Bcl-2 serine70 dephosphorylation induced by palytoxin is mediated through protein phosphatase 2 activation in U937 cells

(A) Palytoxin induces a dose-dependent dephosphorylation Bcl-2 at serine70 in U937 cells. Western blot is representative of three-independent experiments. Band intensities were quantified using ImageJ software. Paclitaxel (500nM, 8h) is used as positive control for multi-site phosphorylation of Bcl-2. ***P<0.005 versus control.

(B) Okadaic acid, a protein phosphatase 2A (PP2A) inhibitor rescues U937 cells from

palytoxin-induced cell death. Hydroquinone (10μM, 24h) was used as positive control for PP2A activation. Cells were stained with Hoechst in order to analyze the nuclear morphology merged with phase contrast images using ImageJ. The percentage of cell death was evaluated by counting the number of cells undergoing various types of cellular death. Three independent fields of 100 cells were quantified. Data representative of three independent experiments are shown with mean ± SD. *$p<0.05$, ***$p<0.005$ versus control.

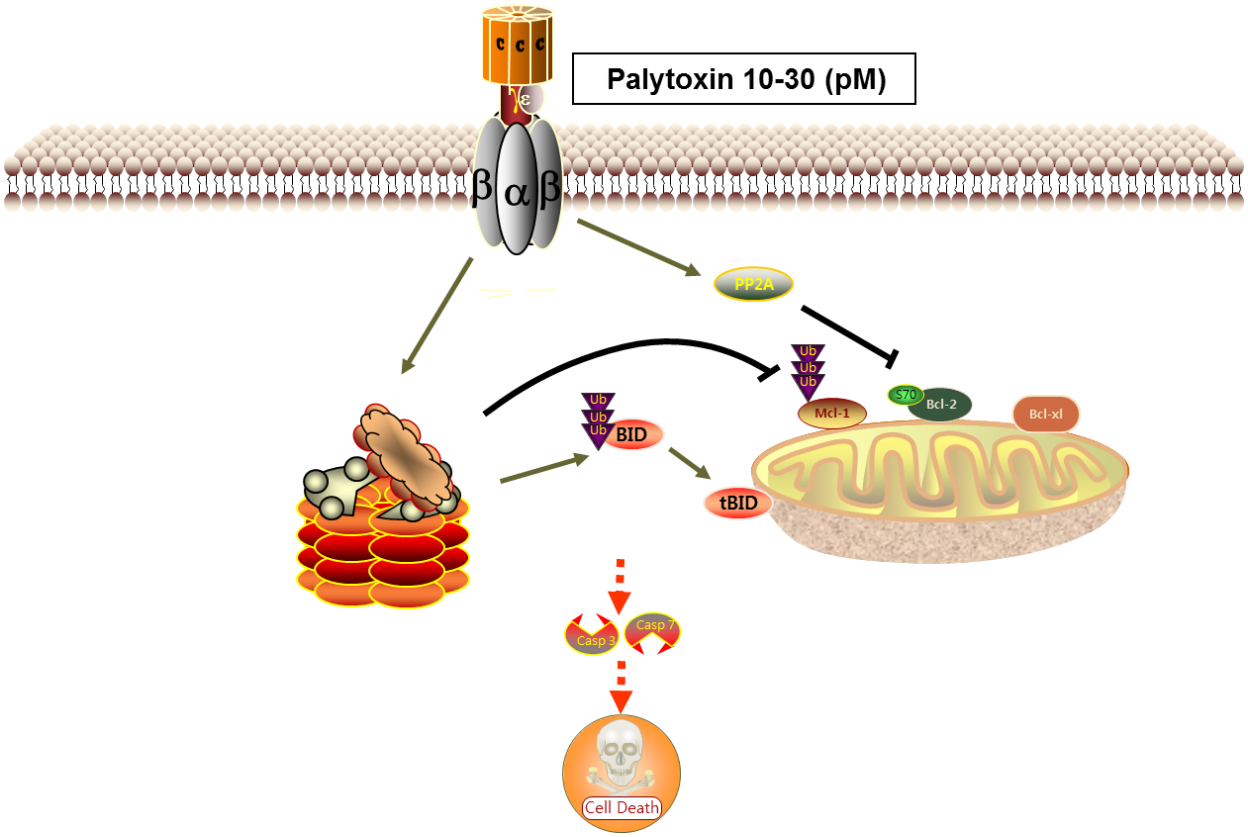


Figure 7. Schematic model of palytoxin-induced cell death

Altogether, palytoxin acts as potent anti-leukemic agent at sub-tumor promoting concentrations, which leads to apoptotic cell death at IC_{50}s at low picomolar concentrations in various leukemia cell modes with reduced differential and systemic toxicity.

To achieve cell death, anti-apoptotic Mcl-1 and Bcl-xL proteins is degraded in a dose-dependent manner. Mcl-1 is ubiquitously degraded by proteasomal up-regulation. Pro-apoptotic BID is truncated and Bcl-2 serine70 is dephosphorylated by PP2A activation leading to a rapid onset of caspase-dependent apoptosis in human leukemia cells.

DISCUSSION

Here we demonstrated that palytoxin, one of the most toxic algal biotoxins known [23], is able to significantly induce apoptotic cell death at low picomolar concentrations in different types of leukemia. We focused our efforts on cell death mechanisms especially in acute myeloid leukemia (AML) U937 cells. Recent research showed that AML has abnormalities in one or more apoptotic pathways leading to a survival advantage over healthy cells [24]. Indeed, palytoxin induced the most common and well defined form of programmed cell death in AML, apoptosis, a physiological process of cellular suicide in a caspase-dependent manner [25]. However, as we observed a small measureable amount of non-apoptotic cell death after caspase inhibition by zVAD, it is essential to determine in the future which type of secondary cell death mechanism the cell activates in the absence of apoptotic capacity [26]. In that sense investigation of activation of non-caspase proteases inducing calpains or cathepsins could be of interest [27], [28].

Among Bcl-2 family proteins, Mcl-1 and Bcl-xL was found to be strongly down-regulated by palytoxin even at low picomolar concentrations. As we observed a consistent and early onset of BID truncation, the differential effect of extrinsic versus intrinsic cell death pathway is of interest and will be investigated by pathway specific caspase inhibitors [17].

We have demonstrated that palytoxin regulates a rapid down-regulation of Mcl-1 protein without involving caspases-dependent modulation but rather a proteasome-dependent degradation of Mcl-1. Thus three major proteasome activities are up regulated by palytoxin possibly leading ubiquitin E3 ligases Skp Cullin F-box containing F-box WD repeat domain containing 7 (SCF^{Fbw7}), β-transducin repeat containing protein 1 (SCFβ-TrCP), and Mcl-1 ubiquitin ligase E3 (Mule) [29], [30] promoting Usp9x (ubiquitin-specific peptidase 9, X-linked) required for Mcl-1 deubiquitination [31]. Moreover, Bcl-2 homology domain-3 (BH-3) only proteins including p53 up-regulated modulator of apoptosis (PUMA) or Phorbol-12-

myristate-13-acetate-induced protein 1 (NOXA) induces degradation of Mcl-1 at the mitochondria level which also requires the E3 ligase Mule responsible for the polyubiquitination Mcl-1 [32]. The implication of NOXA in the regulation of Mcl-1 proteasomal degradation will support our hypothesis as disruption of 26S proteasome function by various mechanism trigger the rapid accumulation of proteasome-dependent BH-3 only family and subsequent cell death induced by apoptosis [32].

It will be interesting to investigate overexpression of Mcl-1 with plasmid constructs more specifically wild type and proteasome resistant isoforms of Mcl-1 [33]. Overexpression of these proteins will further allow us to strengthen the hypothesis of proteasomal degradation. Similarly, stably transfected Jurkat T cells expressing a form of mutated Bcl-2 (serine70, serine87, and threonine 69) will allow us to understand the role of Bcl-2 expression regulated by palytoxin in various models [34], [35].

In this study, we also showed that inhibition of PP2A activity rescued palytoxin-induced cell death, which implies that phosphatases play a major role in cellular mechanisms activated by palytoxin [36]. PP2A has been described as a potential therapeutic target in Philadelphia chromosome-positive chronic myeloid leukemia or in acute myeloid leukemia where PP2A inactivation is a recurrent event [36]. Palytoxin could restore PP2A or related phosphatases to induce caspase-dependent apoptosis mediated through AKT and ERK1/2 pathways [37]. In general, PP2A exerts both inhibitory and stimulatory effects on MAP kinase signaling pathways [38]. Previous findings suggest that palytoxin activates mitogen-activated protein kinase (MAPK) cascade including the three major groups, extracellular signal regulated protein kinases 1 and 2 (ERK1/2), c-Jun N-terminal kinases/stress-activated protein kinase (JNK), p38 protein kinase in response to heat shock protein 27 (HSP27) phosphorylation in human breast adenocarcinoma (MCF-7) cell line [39]. In monocyte-derived human macrophages, palytoxin phosphorylates p38 MAPK and activates nuclear factor kappa B

(NF-κB) pathways by increasing the mRNA levels of inflammation-related genes like interleukin 8 (IL-8) and inhibitor of kappa alpha (IκB-α) leading NF-κB nuclear translocation [6]. These observations have led us to hypothesize that palytoxin may activate a pro-inflammatory signaling cascade in leukemia cell lines by regulation of kinases and phosphatases implicated in the inflammatory process [40].

As palytoxin was described as a tumor promoter even though at higher concentrations [41], we intend to combine palytoxin at subtoxic concentration in co-treatment with known chemotherapeutic and experimental drugs including FMS-like tyrosine kinase 3 (*FLT*3) inhibitor [42], demethylating agents [43], and Bcl-2 family inhibitor in order to synergize anti-leukemic effects induced by apoptotic pathways [44].

In conclusion, we demonstrate here the marine compound palytoxin rapidly induces cell death through apoptosis in human leukemia cell lines. While down regulating the expression of Mcl-1, palytoxin activates BID truncation eventually leading to intrinsic apoptotic pathway are caspase-dependent cell death mechanism. While additional analysis remains to be elucidated as various phosphatases could be involved, these findings show for the first time that palytoxin acts as an apoptotic inducer affecting leukemia cells, which makes palytoxin a promising candidate for new potential anti-leukemic drugs.

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요 약

해양 천연물질은 독특한 유기화합구조로 구성 되어있으며 강력한 생리활성 효능을 가지고 있어 항암신약 개발 물질로서 우수한 개발가치를 평가받고 있습니다. 그중 해양 천연물 비펩디드성 물질로 가장 강력한 독성 효과를 나타내는 Palytoxin 물질이 기존 보도된 연구에 의하면 Na+/K+-ATPase 구조에 결합하여 이온 농도를 변화시킴으로 세포 신호전달을 조절하는 연구가 보도되었습니다.

본인 이번 연구를 통하여 Palytoxin 물질이 다양한 백혈평 세포주들의 항암 억제효과를 나타냄을 창출하였으며, 특별히 정상세포 Peripheral blood mononuclear (PBMC) 및 제브라피시 모델에서도 안정적인 독성효과를 나태냄으로 본 물질이 암세포를 선택적으로 사멸시키는 효과를 발굴하였습니다.

Palytoxin에 의한 항암사멸 효과를 규명하기 위해 신호전달을 연구한 결과, 본 물질이 암세포 유도의 관련된 Caspases 단백질 및 효소 물질에 의하여 세포 사사멸 초래하는 것을 발견하였습니다. 그 중 암세포 생존과 사멸을 조절하는 MCl-1, Bcl-xl 단백질들을 포로테아좀의 기능을 촉진시킴으로 분해하는 효과를 규명하였으며, Bcl-2 serine70 단백질 구조의 PP2A에 탈인산화 기능으로 의하여 생존을 억제하는 효과를 규명하였습니다.

결론적은로 본 연구를 통해 Palytoxin 천연해양물질이 항암신약으로서 우수한 효능을 가지고 있음을 증명하고, 낮은 농도에서도 우수한 약리독성 안정성을 나타내는 물질임을 연구를 통하여 증명하게 되었습니다.

주요여: 해양천연물, Palytoxin, 세포사멸, 백혈병

학번:2013-23458