A Novel Cycloheptapeptide Exerts Strong Anticancer Activity via Stimulation of Multiple Apoptotic Pathways in Caspase-3 Deficient Cancer Cells

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Abstract. Background: VR3848 is a novel cycloheptapeptide, isolated from a Euphorbiaceae plant, which can suppress proliferation of various tumor cells at nanomolar concentration. Due to its novelty and potency, the molecular process of tumor cell growth inhibition was investigated intensively. Materials and Methods: MCF-7 cells, a caspase-3 deficient cancer cell line, were treated with VR3848. The genetic response was monitored using cDNA array analysis. Results: Expression alterations of caspase, bcl-2 family members, death receptor, death adaptor, death ligands, stress response, cell cycle machinery, mitogen-activated protein kinases (MAPKs) and proto-oncogene were found which can be linked into three apoptotic pathways. The first was the death receptor-mediated pathway, which was confirmed by functional inhibition of caspase-8 and -10. The second pathway was via ER-stress apoptosis demonstrated by up-regulation of ER-stress genes and the release of caspase-12 into the cytoplasm. The third pathway involved mitochondrial membrane leakage which was elucidated by down-regulation of anti-apoptotic bcl-2 and an increased level of cytosolic apoptosis-inducing factor (AIF). Cell cycle arrest was observed which may have been a direct effect of VR3848 or a consequence of DNA strand breaks which in turn stimulated cell cycle arrest. Conclusion: VR3848 inhibited MCF-7 cancer cell growth through an activation of three related apoptotic pathways.

The battle against cancer, especially against drug-resistant cancerous cells, is challenging. Various strategies have been developed to overcome this problem (1, 2). One of these approaches is the search for new chemotherapeutic compounds which affect an unexplored cellular target. As part of an ongoing attempt to identify potential chemotherapeutic compounds from tropical plants in Thailand, a novel compound named VR3848 was discovered. This compound was purified from a Euphorbiaceae plant and has been structurally elucidated as a 7-mer cyclic heptapeptide. VR3848 exerts a strong characteristic chemotherapeutic activity, killing cancerous cells at nanomolar concentrations (3, 4). Moreover, VR3848 is 10-fold more potent than vinblastine in the killing of the Lu-1 non-small cell lung cancer cell line (4). In addition, VR3848 exerted a potent cytotoxicity in vivo against human cancer cells KB, Col-2 and LNCap using hollow fiber xenografts (V. Reutrakul, personal communication). Therefore, it is interesting to investigate the molecular mechanism of the anticancer activity of VR3848.

Apoptosis is a mode of cell death which is induced by various types of anticancer agent (5, 6). It is the death of an individual cell and is genetically regulated. Two major apoptotic pathways, intrinsic and extrinsic pathways, have been identified and studied extensively. The extrinsic pathway involves two major organelles, mitochondria and endoplasmic reticula, and death stimuli are generated intracellularly (8-12). The common mediators for both extrinsic and intrinsic pathways are bcl-2 protein family, caspases, cytochrome C, AIF, endo G, GADD 153, JNK (8-12).

Another group of genes that regulates cell survival and cell death is the group of genes involved in cell cycle machinery. These genes serve to protect cells from genotoxic stress. Cyclin and specific cyclin-dependent kinase, CDK, are two major components whose interaction drives cells forward through the cell cycle (13, 14).
Inhibition of the interaction between cyclins and CDK results in cell cycle blocking at both G1- and G2-phases (15, 16). Without repair, cycling cells with damaged DNA will be recognized by the apoptotic machinery and apoptosis will be induced.

The objective of the present study was to elucidate the anticancer mechanism of VR3848. MCF-7, a caspase-3-deficient breast cancer cell line, was selected as a model. This cell line has been widely used for anticancer research due to its lack of functional cardinal caspase, caspase-3, which may be its endogenous characteristic of drug resistance (17). MCF-7 cells were treated with VR3848 and alteration of apoptotic gene profiling was investigated using cDNA array analysis.

Materials and Methods

Reagents and cell lines. VR3848 was isolated from a Euphorbiaceae plant in Thailand using bioassay-directed fractionation. The breast cancer cell line, MCF-7, was purchased from ATCC (ATCC number HTB-22). Cells were grown in Eagle’s minimal essential medium (MEM, Gibco BRL, Grand Island, NY, USA) with L-glutamine, 0.1 mM non-essential amino acids, 1 mM pyruvate (Gibco BRL, Grand Island, NY, USA), 0.01 mg/ml bovine insulin (Sigma Chemical Co. Ltd., St. Louis, MO, USA) and 10% of heat-inactivated (56°C, 30 min) fetal bovine serum. The bovine insulin (Sigma Chemical Co. Ltd., St. Louis, MO, USA) and pyruvate (GIBCO BRL, Grand Island, NY, USA), 0.01 mg/ml L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate (GIBCO BRL, Grand Island, NY, USA) and 10% of heat-inactivated (56°C, 30 min) fetal bovine serum. The confluent cells were passaged every 3 days.

Cytotoxicity test. MCF-7 cells were treated with VR3848 at the concentration of 0.1, 1.0, 10 and 100 nM for 72 h. The cytotoxic effect of VR3848 on MCF-7 cells was detected using an SRB assay as described elsewhere (18). The GI50, the concentration producing 50% inhibition of cell growth, of VR-3848 was subsequently obtained using non-linear regression analysis. The data are means of three independent experiments. Vinblastine and 0.1% DMSO, a diluent of VR3848, were used as control compounds.

Apoptosis detection. Monolayer cultures of MCF-7 cells were treated with 100 nM VR3848. At 6, 12, 18 and 24 h of treatment, cells undergoing apoptosis were detected using DAPI staining and a TUNEL assay as described elsewhere (19). The number of apoptotic cells were counted under UV microscopy after DAPI staining. A total of 500 cells were blindly counted by two independent investigators. The number of apoptotic cells was expressed as a percentage.

Screening of apoptosis-related gene expression using a cDNA macroarray. MCF-7 cells were treated with 100 nM VR3848 for 6 h. Total RNA was isolated from VR3848-treated cells and control cells using the Nucleospin® RNA II kit (Macherey-Nagel, Duren, Germany) in accordance with the manufacturer’s instructions. Approximately 5 µg of RNA was used immediately for poly A+ RNA enrichment using oligo-dT beads as recommended by the manufacturer. The poly A RNA was reverse transcribed and labeled with 32P-dATP. The 32P-labeled cDNA from VR3848-treated cells and control cells was used to probe BD Atlas® human apoptosis array (Clontech Laboratories, Palo Alto, CA, USA). The complete list of genes can be obtained from www.clontech.com. Hybridization was carried out according to the manufacturer’s instructions. Data acquisition and analysis were performed as described elsewhere (19).

RT-PCR-based gene expression analysis. Total RNA of MCF-7 treated cells and control cells at each time-point was isolated using Nucleospin column (Clontech). RNA was then subjected to first strand cDNA synthesis before being further amplified by PCR using specific primers as described elsewhere (19). GADD 153 gene primers were sense 5’GAA ACG GAA ACA GAG TGG TCA TTC CCC 3’ and antisense 5’ GTG GGA TTG AGG GTC ACA TCA TTG GCA 3’. DR5 (TRAIL receptor) gene primers were sense 5’ CAC CAG GTG TGA TTC AGG TG 3’ and antisense 5’ CCC CAC TGT GCT TGT TAC CT 3’. CDK4 gene primers were sense 5’ GCC CCT CTA GAA CAG GAG AG 3’ and antisense 5’ TAC CTT GAT CTC CCG GTC AG 3’. CDK5 gene primers were sense 5’ GTC CAT CTA CAT GTG TGC AG 3’ and antisense 5’ CTC CTT GTG GTA TTG AGT TT 3’. ERK-1 gene primers were sense 5’ ACA GAG TCT GCC CTC CA 3’ and antisense 5’ CTC ATC CTT CGG GTC ATA GT 3’. Human β-actin gene primers were sense 5’ ATC TGG CAC ACT CT TCA AGT 3’ and antisense 5’ GCT TGG AGT ATG CCA CAG TCA 3’. The level of gene expression was semi-quantified using densitometry and expressed as percentage of a ratio relative to that of β-actin, an internal control.

Apoptosis inhibition by specific caspase inhibitors. Cell suspensions (5x104 cells/well) were plated into 24-well plates and incubated for 29 h before treatment. Cells were pretreated with inhibitors, caspase-8 (Z-IETD-FMK, R&D system) or caspase-10 (Z-AEVD-FMK, R&D system) or caspase-8 plus caspase-10 inhibitors for 2 h. After pretreatment, cultures were treated with 100 nM VR3848 in the presence or absence of inhibitors. Caspase-8 and caspase-10 inhibitors at a concentration of 20 µM and 40 µM were used, respectively. Numbers of apoptotic cells were determined using DAPI staining at 6, 12 and 18 h.

Detection of apoptosis-inducing factor (AIF) and caspase-12 using Western Blotting. VR3848-treated cells or control cells were harvested at 6, 12, 18, 24 and 36 h. The cytosolic fractions were extracted from harvested cells as described elsewhere (20). These samples were then subjected to the immunoblotting technique using rabbit-anti-human AIF antibody (R&D Systems, Inc., Weisbaden, Germany) or rabbit-anti-human caspase-12 antibody (CHEMICON International Inc., Temecula, CA, USA). The level of protein was semiquantified using a densitometry band analyzer and expressed as a percentage of a ratio relative to actin protein, an internal control.

Results

Growth inhibition effects and apoptosis induction in VR3848-treated MCF-7 cells. The anti-cancer potency of VR3848 was expressed as the 50% growth inhibitory value (GI50). Vinblastine was used as a reference drug. The GI50 value for VR3848 and vinblastine were 8.2 and 1.5 nM, respectively, indicating that the antiproliferative effect of VR3848 was 5.3-fold less potent than vinblastine for MCF-7 cells.
Nuclear morphological changes of VR3848-treated MCF-7 cells were detected using DAPI and TUNEL assay. As illustrated in Figure 1 A and B, nuclear condensation and fragmentation were clearly found using DAPI staining, while chromosomal strand breaks were also detected with the TUNEL assay. This data indicated that VR3848 inhibited growth of MCF-7 cells through apoptosis. The number of cells undergoing apoptotic death increased depending on the duration of treatment (Figure 1C).

Identification of apoptotic genes response to VR3848 growth inhibition. To perform a reliable cDNA array analysis,

Figure 1. Detection of apoptosis in MCF-7 cells induced by 100 nM of VR3848. DNA strand break and nuclear fragmentation were detected with a TUNEL assay and DAPI staining, respectively. (A) TUNEL staining: arrow indicates nuclei with a DNA strand break. (B) DAPI staining: nuclear fragmentation and condensation were detected, indicated by arrows. (C) Numbers of apoptotic cells at 6, 12, 18 and 24 h are presented as percentages as mentioned in Materials and Methods.
reproducibility of the technique must be tested. A single set of purified RNA sample was divided into two sets of RNA, labeled and used as probes to hybridize with the array membrane. The hybridization intensities of each gene on the two arrays were plotted against each other (data not shown). The correlation coefficient between the two data sets was 0.89 (p≤0.05). This data indicates the high reproducibility with this method.

Apoptotic genes with a mean level of expression that was 2-fold higher or lower in the treated cells compared to the control were selected and are listed in Table I. Genes of caspases, death receptors, death ligands, death adaptors and ER-stress responses were strongly up-regulated. Genes that regulate cellular proliferation and differentiation such as cyclins, cell cycle-dependent kinase, proto-oncogenes and MAPK pathway-related genes, were both up- and down-regulated in response to VR3848 treatment. An alteration of the expression of the members of the bcl-2 family was also detected.

Validation of cDNA array analysis. According to the genes listed in Table I, genes involved in two major pathways, the receptor/ligand mediated pathway and the ER-stress pathway, were up-regulated, suggesting that death in response to VR3848 may be mediated through these pathways.

For receptor/ligand mediated pathways, the major mediators are death receptors, death ligand, caspase 8 and caspase 10. To confirm the role of this pathway in VR3848-induced death, up-regulation of DR5 or TRAIL-receptor gene was evaluated with RT-PCR and the role of caspase-8 and -10 were tested using caspase-specific inhibitors. As demonstrated in Figure 2A, the expression of the TRAIL gene was significantly up-regulated after 6 h of treatment and continued increasing up to 24 h.

Caspase-8 and -10 are upstream caspases which rapidly respond to signals from death ligand/receptor interaction. To further confirm the role of receptor-mediated apoptosis induced by VR3848, the activities of caspase-8 and -10 were blocked by specific inhibitors. Both caspase-8 and -10 inhibitors were able to suppress apoptosis during early treatment, up to 18 h (Figure 2B). These data indicated that death induced by VR3848 involved a receptor/ligand mediated pathway.

To validate participation of the ER-stress pathway, the up-regulation of the ER-stress response gene, GADD 153 and caspase-12 were semi-quantified by RT-PCR and Western blot, respectively. The expression of the GADD 153 gene was significantly up-regulated after 6 h of treatment and continued increasing up to 24 h.

To validate participation of the ER-stress pathway, the up-regulation of the ER-stress response gene, GADD 153 and caspase-12 were semi-quantified by RT-PCR and Western blot, respectively. The expression of the GADD 153 gene was significantly up-regulated after 6 h of treatment and continued increasing up to 24 h.

To validate the role of the cell cycle machinery and MAPKs, the levels of CDK4, CDK5 and ERK-1 gene expression were determined using semi-quantitative RT-PCR. As demonstrated in Figure 4, expressions of these three genes were down-regulated.

Detection of the caspase-independent mitochondrial pathway. MCF-7 cells lack functional caspase-3 (17). Therefore, the caspase-independent mitochondrial pathway was
investigated. An up-regulation of a representative of this pathway, AIF, was monitored in VR3848-treated MCF-7 cells using a specific monoclonal antibody. Release of AIF into the cytoplasm was detected at 12 h after treatment. The higher amount of cytoplasmic AIF correlated with the duration of VR3848 treatment (Figure 5). These data indicated that VR3848 induced the caspase-independent mitochondrial pathway.
Discussion

VR3848, a novel 7-mer cycloheptapeptide isolated from an Euphorbiaceae plant, inhibits growth of caspase-3-deficient breast cancer cell of the MCF-7 cell line. This anticancer characteristic was modulated through a selective trigger of apoptosis which was evidenced by nuclear morphological changes such as nuclear condensation/fragmentation and chromosomal strand breaks. Our data are supported by previous reports in which VR3848 is an apoptosis-inducing natural peptide (3, 4). The anticancer potency of VR3848 was found to be in the nanomolar concentration range of 5, 8.2 and 10 nM for human leukemia, MCF-7 and human lung cancer cells, respectively (3, 4). The anticancer potency of VR3848 is comparable to vinblastine, a chemotherapeutic drug. For example, VR3848 is 10-fold more active on Lu-1 cells than vinblastine, while 5-fold less potent than vinblastine for MCF-7 cells. These data suggested that VR3848 and vinblastine target different molecules. Due to its novelty and its potency, the anticancer mechanism of this compound deserves further exploration.

An extrinsic pathway or receptor/ligand-mediated apoptotic pathway begins outside a cell when conditions in the extracellular environment determine that a cell must die. This pathway is extensively studied in FASL-FAS, TNF-TNFRI systems. Interaction of FASL with FAS recruits upstream caspases, caspase-8 and -10, through the function of an adaptor protein called FAS-associated death domain (FADD) (21). These caspases then directly stimulate downstream executioner caspases or induce apoptosis through the mitochondrial pathway by damaging the balance of the bcl-2 system (21). VR3848 stimulated several types of death ligand gene, death receptor gene and caspase-8 and -10 in MCF-7 cells. These data led us to hypothesize that receptor/ligand-mediated apoptosis may be one of the suicidal pathways committed by MCF-7 cells in response to VR3848 treatment. This hypothesis was proven in the present study in that caspase-8 and -10 inhibitors were able to suppress apoptotic death in MCF-7 during the early phase of VR3848 treatment. In addition, our cDNA array screening demonstrated down-regulation of Daxx, an anti-Fas-mediated apoptotic protein (22-24). These data indicated that VR3848 treatment may facilitate apoptosis via receptor/ligand interaction. Receptor-mediated apoptosis was also demonstrated in prostate tumor cells by depsipeptide (FR901228) (25).

The mitochondrion is a major cytoplasmic organelle that participates in apoptosis. An imbalance between pro-apoptotic bcl-2 and antiapoptotic bcl-2 proteins causing damage to the mitochondrial membrane potential results in release of caspase-dependent and caspase-independent apoptotic mediators from the mitochondrial membrane space (8, 9). Our investigation found significant increases in the level of cytoplasmic AIF in response to VR3848 treatment, indicating that VR3848 induced the caspase-independent mitochondrial pathway in MCF-7 cell apoptosis. In addition to previous study in human leukemia cells, we concluded that VR3848 stimulated both caspase- and non-caspase mitochondrial-dependent apoptosis depending on the type of tumor cell (3). Other types of natural products such as carbazole alkaloid from vegetables, cyclic depsipeptide AT514 from bacteria and cyclic dipéptide are able to stimulate mitochondrial-dependent apoptosis via cytochrome C and caspase-3 activation (26-28).

The endoplasmic reticulum is another cytoplasmic organelle that participates in apoptosis via caspase-4 and -12, GADD153 and JNK. During VR3848 treatment, strong up-regulation of GADD153, GADD45, GSTT1 and release of caspase-12 into the cytoplasm were detected, suggesting that VR3848-induced death involved the ER-stress system.

Another group of genes that responded to VR3848 treatment were the genes involved in the cell cycle machinery including cyclins, cell cycle-dependent kinases and the MAPK system. Various types of natural products inhibit proliferation of cancer cells by induction of cell cycle arrest (29-33). During VR3848-induced death in MCF-7, G2-specific cyclins (cyclin A, B1, G1), CDK4 and MAPK-related genes (GRB2, ERK1,2,4, MAPKK3) were down-regulated, suggesting that death of MCF-7 in responding to VR3848 involved cell cycle arrest. The involvement of the cell cycle in this death was accompanied by suppression of CDK-5, which has been demonstrated to serve as an antiapoptotic factor (34, 35). Whether VR3848 targeted the cell cycle machinery or an alteration of the cell cycle was a consequence of DNA strand breaks requires further investigation.

In the present study, our results indicated that VR3848, a novel cycloheptapeptide, induced apoptosis in a caspase-3-deficient breast cancer cell line via multiple pathways and various cytoplasmic organelles. This indicates a potential role of VR3848 in cancer therapy. The precise target of action of VR3848 remains to be elucidated.

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References


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