RESEARCH ARTICLE

Editorial Process: Submission:03/28/2023 Acceptance:08/19/2023

Endoplasmic Reticulum Stress-Mediated Apoptosis Induced by VR12684 Isolated from *Mallotus spodocarpus* in Cholangiocarcinoma Cell Line

Chariya Hahnvajanawong[1,2], Wissanukorn Puthabaln[1,3], Thidarut Boonmars[2,4], Vichai Reutrakul[3,5], Parichart Boueroy[6]*

Abstract

Introduction: Cholangiocarcinoma (CCA) is a poor prognosis of a malignant tumor that has been unresponsive to conventional chemotherapeutic agents. Effective and novel therapeutic agents are urgently needed. VR12684 (isolated from *Mallotus spodocarpus*) has been reported to exhibit growth inhibitory activities in cancer cell lines. The present study investigated the growth inhibitory mechanisms of this compound in a human CCA cell line (KKU-M156). **Methods:** The effects of VR12684 on anti-proliferation, cell cycle arrest and apoptosis induction in CCA cells were demonstrated by SRB assay, flow cytometry, acridine orange/ethidium bromide (AO/EB) staining and western blot analysis. **Results:** Treatment with VR12684 decreased cell proliferation in a dose- and time-dependent manner in the KKU-M156 cell line. VR12684 induced cell cycle arrest in the G2 phase in KKU-M156 through down-regulation of cyclin B1 and Cdk1 and up-regulation of p21, p27 and p53 levels. VR12684 induced mitochondria-mediated apoptosis by increasing DNA fragmentation, the Bax/BCL-2 ratio and AIF, and decreasing survivin with subsequent activation of caspase-9 and -3. This compound could induce apoptosis through the endoplasmic reticulum (ER) stress-mediated pathway by up-regulation of GRP78, IRE1α and GADD153 levels leading to down-regulation of Bcl-2 and activation of calpain-1, caspase-7 and -12. **Conclusion:** These results suggested that VR12684 inhibited KKU-M-156 cell growth by way of cell cycle arrest and induction of apoptosis, at least in part, through the mitochondria- and ER-associated intrinsic pathways. Such compounds warrant evaluation as a candidate for the treatment of human CCA.

Keywords: Endoplasmic reticulum pathway- mitochondria-mediated apoptotic pathway- *Mallotus spodocarpus*

Asian Pac J Cancer Prev, **24 (8)**, 2741-2748

Introduction

Cholangiocarcinoma (CCA) is a malignant tumor of the bile duct epithelium. The worldwide incidence of CCA is 6/100,000 people but is 85/100,000 people in Northeastern Thailand (Banales et al., 2016) where the management cost is USD 120 million annually (Sripa et al., 2012; Loaharanu and Sornmani, 1991). A complete surgical resection with histological negative resection margins is the only curative treatment option for CCA (Bridgewater et al., 2014). Resistance to the chemotherapeutic drugs for CCA is a major challenge and effective pharmacological treatments are still limited.

Mallotus spodocarpus Airy Shaw (traditionally known in Thailand as 'takhe khumwang' or 'tao tua mia') is a traditional plant in central, northern, northeastern and southwestern Thailand and is classify in the family Euphorbiaceae (Uthaisang et al., 2004). The fraction of this plant have been shown to have anti-inflammatory activity on ear edema formation (Intahphuak et al., 2004). Pootrakronchai (2000) reported on the anti-cancer effect of VR-3848, a novel peptide extracted from species in the family Euphorbiaceae against the LU-1 non-small cell lung cancer cell line. Moreover, Uthaisang et al., (2004) reported on the molecular mechanism of VR-3848 on growth inhibition of the leukemic Jurkat cell line by the mitochondria-dependent apoptosis pathway. Ubol et al., (2007) demonstrated that VR-3848 inhibited breast cancer cell line (MCF-7) growth through activation of three related apoptotic pathways consisting of death receptor mediation, demonstrated ER-stress apoptosis and a mitochondrial-mediated pathway. The compound

*[1]Department of Microbiology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand. [2]Cholangiocarcinoma Research Institute, Khon Kaen University, Khon Kaen 40002, Thailand. [3]Center of Excellence for Innovation in Chemistry, Department of Chemistry, Faculty of Science, Mahidol University 10400, Thailand. [4]Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand. [5]Department of Chemistry, Faculty of Science, Mahidol University, Bangkok 10400, Thailand. [6]Faculty of Public Health, Kasetsart University Chalermphrakiat Sakon Nakhon Province Campus, Sakon Nakhon 47000, Thailand. *For Correspondence: parichart.bou@ku.th*

extract VR12684 from *M. spodocarpus* when purified has been structurally elucidated as a novel compound, 7-mer cyclic peptide. Due to VR-3848 potency, the anticancer mechanism of this compound deserves further exploration. Many current clinically used anticancer drugs are known to kill tumor cells through the induction of apoptosis and cell cycle arrest. Therefore, the discovery and development of an inducer of apoptosis and cell cycle arrest could lead to new anticancer agents.

The endoplasmic reticulum (ER) plays a key role in protein folding and trafficking. Cellular stress can cause abnormalities in ER function leading to unfolded and misfolded proteins, which initiate the unfolded protein response (UPR) inside the ER (Song et al., 2018). The ER stress pathway is triggered in three pathways: PERK/eIF2α/ATF4, IRE-1/Xbp-1 and ATF6 activation. ER stress is able to initiate the intrinsic apoptotic pathway, leading to cell death (Xu et al., 2013). However, knowledge is limited of the mechanisms of ER-stress mediated apoptosis which are activated by chemotherapeutic compounds.

Materials and Methods

Plant compound and human cell line

Mallotus spodocarpus compound (VR12684) was kindly provided by Professor Vichai Reutrakul, Department of Chemistry, Faculty of Science, Mahidol University, Bangkok, Thailand. VR12684 was extracted and purified from tropical plant species of the Euphobiaceae in Thailand using bioassay-directed fractionation. Stock solution (1 mg/ml) of VR12684 was solubilized with dimethylsulfoxide (DMSO). The KKU-M156 cell line was established at the Department of Pathology, Faculty of Medicine, Khon Kaen University, Thailand and grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a 5% CO_2 humidified incubator.

Reagents and antibodies

Acridine orange/ethidium bromide (AO/EB) was obtained from Sigma, Chemical Company, St. Louis, MO, USA. Hybond ECL nitrocellulose membrane was obtained from Amersham, Pharmacia Biotech, UK. Anti-Bax, anti-Bcl-2, anti-cdk 2, anti-cdk 4, anti-cyclin A, anti-cyclin D1, anti-cyclin E, anti-GADD153, anti-p21, anti-p27, anti-survivin, HRP-conjugated goat anti-mouse IgG and HRP-conjugated goat anti-rabbit IgG antibodies were purchased from Santa Cruz Biotechnology, CA, USA. Anti-activated caspase-3 and anti-β-actin antibodies were purchased from Sigma, Chemical Company, St. Louis, MO, USA. Anti-caspase-7, anti-caspase-8, anti-caspase-9, anti-caspase-12, anti-IRE-1α, anti-procaspase-3 antibodies were purchased from Cell Signaling Technology, Beverly, MA, USA. Anti-AIF antibody was purchased from R&D Systems, Minneapolis, MN, USA. Anti-calpain 1 antibody was purchased from Abcam, Cambridge, UK.

In vitro growth inhibitory assay

The anti-proliferative effect of VR12684 on the KKU-M156 cell line was determined using SRB assay. Briefly, KKU-M156 cells (1×10^4 cells/ml) were treated in a 5% CO_2 humidified incubator with various concentrations of VR12684 (0.01, 0.02, 0.04, 0.08 µg/ml) for 72 h at 37 °C. The cells were stained with SRB and solubilized with Tris buffer. The absorbance (OD) of each well was measured using ELISA (ELISA Reader, Sunrise, USA) plate reader at 540 nm. The IC_{50} value was calculated from concentration-effect curves after linear regression analysis.

Cell cycle analysis

Cells were treated at the indicated concentrations of VR12684 for 24 h. After that, cells were harvested and fixed with cold 70% ethanol overnight at 4˚C. Before analysis, cells were washed with PBS and incubated with 0.1 mg/ml of RNase A at 37˚C for 1 h, followed by PI staining (50 µg/ml) at 4˚C for 30 min in the dark. Samples were examined using a FACSCantoTM II flow cytometer (BD Biosciences, San Jose, CA, USA) and the data were analyzed using the FACSDivaTM software (BD Biosciences).

Acridine orange/ethidium bromide staining

Apoptosis was determined by the detection of nuclear morphology using AO/EB staining. Briefly, CCA cells ($1x10^4$ cells/well) were seeded in 96-well plates and incubated at 37°C in a 5% CO_2 incubator for 24 h and then were treated with the compound at the appropriate concentration. After incubation at 37 °C for 48 h, the treated cells were stained with 8 µl of 100 µg/ml AO/EB mixture and observed under a Nikon fluorescent microscope (Nikon Corporation, Tokyo, Japan). Apoptotic cells were characterized by condensed or fragmented chromatin and counted from a total of 500 cells (Hahnvajanawong et al., 2014).

Proteins extraction of total cell, cytosol or nuclear lysates

Preparation of total cell and nuclear fractions was carried out according to the procedure of (Wattanawongdon et al., 2015). Briefly, cells ($1x10^6$ cells/dish) were seeded into 100 mm dishes for 24 h and then treated with the various concentrations of VR12684 for the indicated times. For the preparation of total cell lysate, the treated cells were lysed in cold RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, 0.5% Nonidet P-40, 1 mM dithiothreitol, 0.1 % sodium dodesyl sulfate, 0.5 % deoxycholate, 1 mM ethylene diamine tetraacetic acid [EDTA]) supplemented with 1 mM E-64, 2 mM phenylmethylsulfonyl fluoride and 2 mM leupeptin. The preparation of the cytosolic fraction was performed using the method described by (Wattanawongdon et al., 2015) with some modifications. Briefly, the treated cells were lysed with translocation buffer (2 mM EDTA, 20 mM Tris-HCl, 0.5 mM ethylene glycol tetraacetic acid, 0.33 M sucrose, 2 mM phenylmethylsulfonyl fluoride, 1 mM E-64, and 2 mM leupeptin). Membrane proteins were extracted with translocation buffer containing 0.1% Triton X-100, shaken at 4°C overnight, and then centrifuged at 13,000×g 4 °C for 30 min. The protein concentrations were determined using the Bradford method (Bradford, 1976).

Western blot analysis

The proteins were separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electro transferred to nitrocellulose membrane. The membrane was blocked with 5 % non-fat dry milk in 0.1 % Tween 20 in PBS, followed by incubation with the indicated primary antibodies (cyclin A, cyclin B1, Cdk1, p27, p21, p53, Bcl-2, Bax, survivin, AIF, procaspase-9, activated caspase-9, procaspase-3, activated caspase-3, GRP78, IRE1α, GADD153, calpain-1, cleaved calpain-1, procaspase-7, activated caspase-7, procaspase-12, activated caspase-12 and β-actin) at 4 ºC overnight and then incubated with the corresponding HRP-conjugated secondary antibody. Signals were detected using an enhanced chemiluminescence kit and autoradiography with CL-XPosure film. For every immunoblot, an equal loading of protein was confirmed by stripping the blot and reprobing with β-actin antibody. The intensities of the protein bands were quantified using the Scion Image software (Version 4.0.2; Scion Corporation, Frederick, MD, USA).

Statistical analysis

Data were expressed as mean ± standard error (SE). Comparisons between untreated control cells and treated cells were made using Student's t-test in the SPSS statistical software, version 16.0 (SPSS, Chicago, IL, USA). Differences were considered significant at p-value, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. All analyses were performed using the Microsoft Excel computer software (Microsoft, Redmond, WA, USA).

Results

Growth inhibitory effect of VR12684 on KKU-M156 cell line

An in vitro growth inhibitory effect of VR12684 on KKU-M156 cells was investigated using SRB assay. Cells in the exponential growth phase were treated with increasing concentrations of VR12684 (0-0.08 µg/ml) for 6, 12, 24, 48 and 72 h. It was found that VR12684 caused marked growth inhibition against KKU-M156 cells in a dose- and time-dependent manner (Figure 1a) with IC_{50} values at 6, 12, 24, 48 and 72 h treatment of 0.071, 0.053, 0.042, 0.035 and 0.031 µg/ml, respectively.

VR 12687-induced cell-cycle arrest in G2 phase

After treatment with various concentrations of VR12684 for 12 h, the results showed that dysregulation of the cell cycle was one of the mechanisms responsible for cell growth inhibition in the VR12684-treated KKU-M156 cell line (Figure 2b). In KKU-M156 cells, VR12684 induced a significant dose-dependent accumulation of cells in the G2/M phase from 39% to 47% with a significant concomitant reduction of cell in the G0/G1 phase from 49% to 39%. In contrast, the population of cells in the S

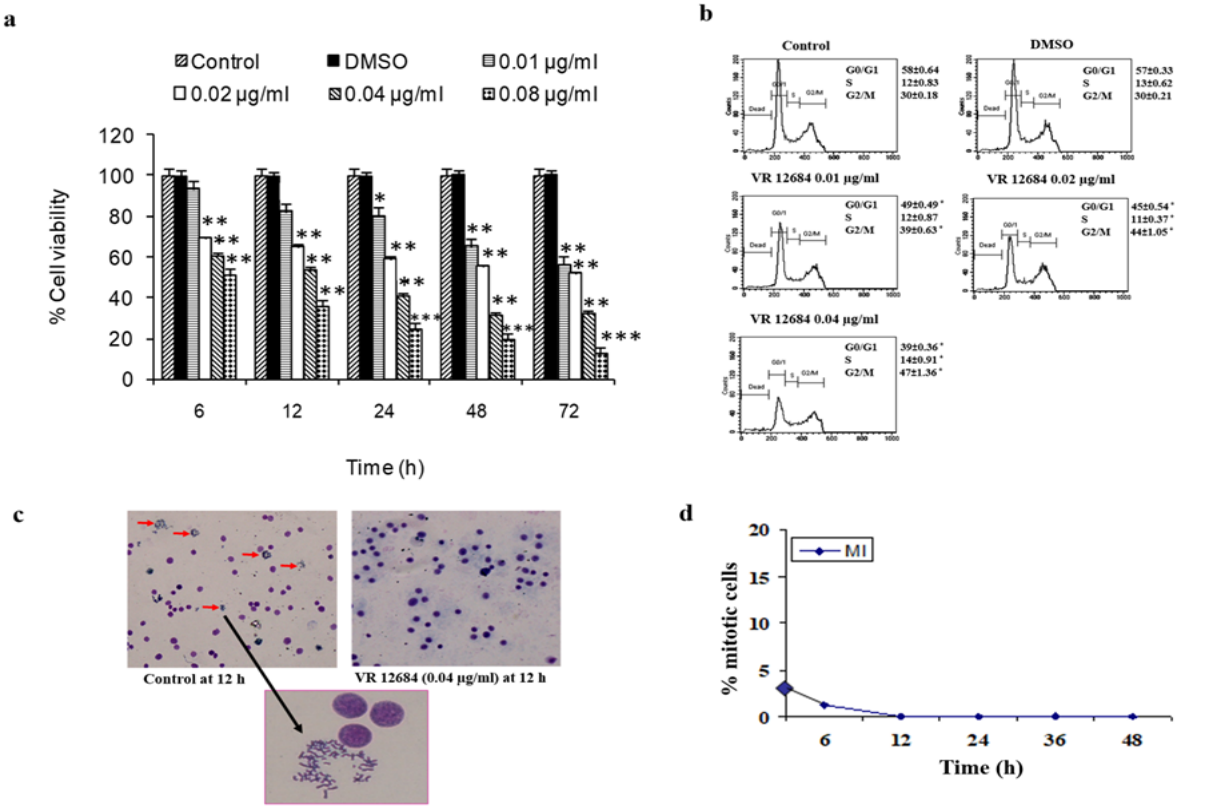

Figure 1. Growth Inhibitory Effect of VR12684 against Human Cholangiocarcinoma Cell Line (KKU-M156). (a) Cell viability was determined using SRB assay. (b) Cell cycle distribution was determined using flow cytometry analysis. (c) Photographs show representative cell populations stained with May Giemsa Grunwald. Mitotic figures could not be detected at 48 h, indicating G2 phase arrest. Cells were in mitosis (arrow). (d) The mitotic index (MI) of KKU-M156 cells. Each value represents mean ± SE of three independent experiments. *p<0.05, **p<0.01, ***p<0.001 versus the 0.1% DMSO-treated cells.

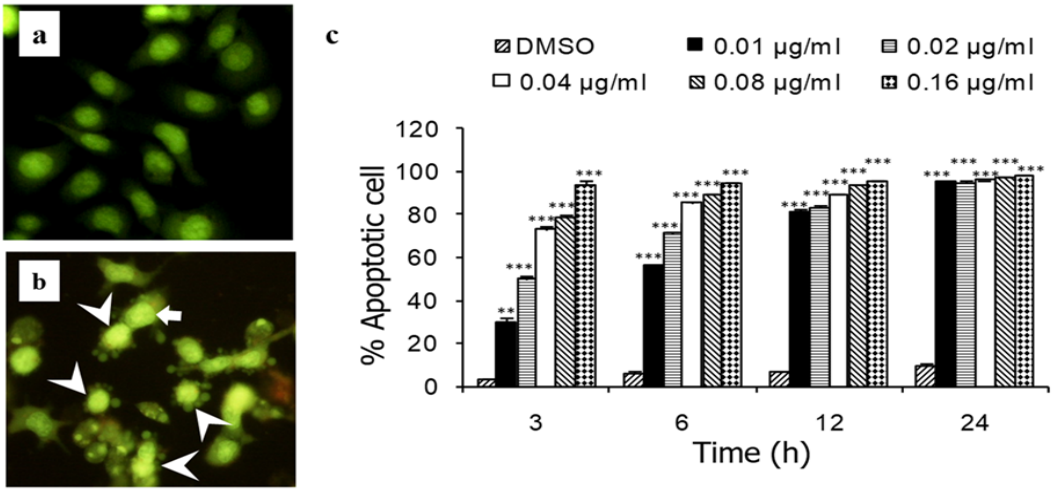

Figure 2. Induction of Apoptosis by VR12684 in KKU-M156 Cells. Cells were treated with vehicle only or various concentrations of VR12684 for indicated times. EB/AO staining: fluorescence photomicrographs (400X) show: (a) DMSO-treated KKU- M156 and (b) VR12684-treated KKU-M156 cells. Nuclei of treated cells show chromatin condensation (arrows) and nuclear fragmentation (arrow heads). (c) Percentages of apoptotic cells of the VR12684-treated and non-treated KKU- M156 cells. Data are mean ± SE of three independent experiments. *p<0.05, **p<0.01, ***p<0.001 versus the 0.1% DMSO-treated cells.

phase did not change significantly (Figure 1b).

Induction of G2 or M arrest by VR12684 in KKU-M156 cells

Cell populations in the G2 and M phases could not be distinguished using FACS analysis. Therefore, the percentage of mitotic cells was calculated to determine whether VR12684 induced a high percentage of M-phase population in the KKU-M156 cells. As shown in Figure 1c and d, the percentage of mitotic cells decreased from 3.2% to 0% at 12 h. Cells in mitosis were not observed up to 48 h. These results indicated that VR12684 induced G2 arrest in KKU-M156 cells.

Apoptosis induction in KKU-M156 by VR12684

Cell morphological changes were observed after treatment of the KKU-M156 cells with various concentrations of VR12684 (0.01-0.16 µg/ml) for 3, 6, 12 and 24 h. Based on AO/EB staining, the nuclei with green homogeneous chromatin distribution could be seen in the vehicle control cells (Figure 2a), while chromatin condensation and nuclear fragmentation (characteristic apoptotic features) were observed in the VR12684-treated cells (Figure 2b). The percentages of apoptotic cells substantially increased in a dose- and time-dependent manner when cells were treated with VR12684 (Figure 2b and c). It was evident that VR12684 induced apoptosis in KKU-M156.

Effect of VR12684 on expression of cell cycle regulators

In KKU-M156 cells, the VR12684 treatment resulted in G2 phase arrest; thus, the expression of G2/M regulators cyclin A, cyclin B1, Cdk1, p21, p27 and p53 were analyzed. In G2/M phase arrest, VR12684 significantly decreased protein expression of cyclin B1 and Cdk1, whereas p21, p27 and p53 levels were significantly increased but had no effect on the expression of cyclin A (Figure 3a and b).

Mitochondria-mediated apoptotic induction induced by VR12684

To further investigate the mechanisms of VR12684 on the induction of apoptosis, we examined the effect of this compound on the level of apoptosis regulatory protein expressions. KKU-M156 cells were treated with 0.01, 0.02 and 0.08 µg/ml of VR12684 for 12 h and the levels of protein expressions were examined. As shown in Figure 5, VR12684 significantly decreased the protein level of Bcl-2, whereas it increased the protein level of Bax in a dose-dependent manner. VR12684 decreased procaspase-9 and procaspase-3 while it significantly increased activated caspase-9 and activated caspase-3 in a dose-dependent manner (Figure 3c and d). Survivin has been reported to be an anti-apoptotic molecule by binding to activated caspase-3 and -7 (Woo et al., 2013). Treatment with VR12684 significantly decreased survivin protein expression in a dose-dependent manner (Figure 3c and d). The apoptosis inducing factor (AIF) protein plays an important role in the mitochondrial-related caspase-independent pathway, so we considered whether the AIF protein might be involved in cell death induced by VR12684. VR12684 treatment resulted in a significantly dose-dependent increase in the AIF protein (Figure 3c and d). Therefore, one important mechanism of VR12684-induced apoptosis in KKU-M156 was via the mitochondria-mediated apoptosis pathway.

Endoplasmic reticulum stress-mediated apoptotic induced by VR12684

The expression of ER stress-related proteins was examined to determine the effect of VR12684 on the ER stress-mediated apoptosis pathway. As shown in Figure 4a and b, VR12684 treatment significantly increased levels of the GRP78, IRE1α and GADD153 proteins in a dose-dependent manner. ER stress-mediated apoptosis involves initiator or executioner caspases, including caspase-12, -7, and -3 (Kerbiriou et al., 2009; Rao et

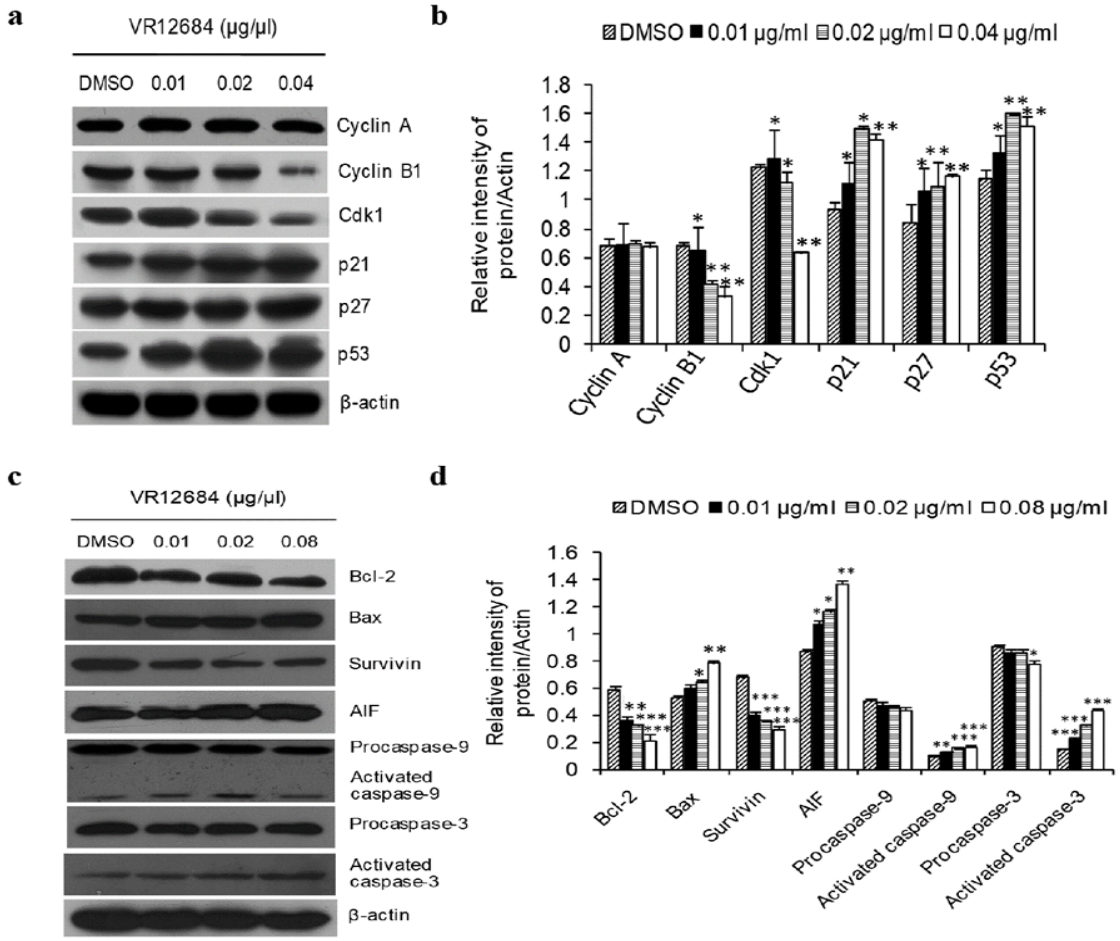

Figure 3. Effect of VR12684 on the Expression of Cell Cycle and Mitochondria-Mediated Apoptosis Regulatory Proteins in KKU-M156 Cells. Total cell lysates were prepared, and 50 μg of protein was subjected to SDS-PAGE followed by Western blot analysis and chemiluminescence detection. (a) Representative protein bands of cell cycle regulatory proteins. (b) Histograms showing the density of cell cycle regulatory protein normalized to β-actin protein of KKU-M156 cells. (c) Representative protein bands of mitochondria-mediated apoptosis regulatory proteins. (d) Histograms showing the density of mitochondria-mediated apoptosis regulatory proteins normalized to β-actin protein of KKU-M156 cells. Each value represents mean ± SE of three independent experiments. *p<0.05, **p<0.01, ***p<0.001 versus the 0.1% DMSO-treated cells.

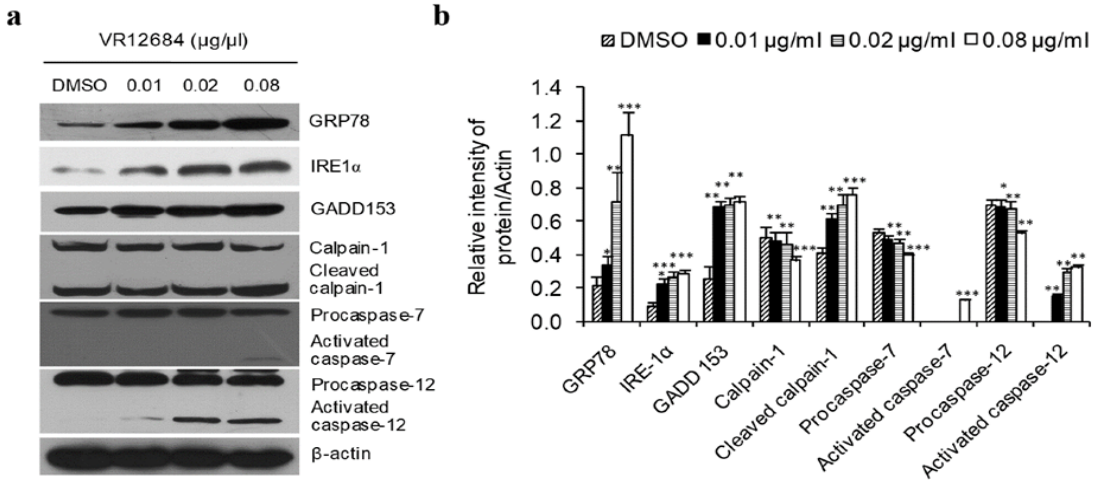

Figure 4. Effect of VR12684 on the Expression of ER Stress-Mediated Apoptosis Regulatory Proteins in KKU-M156 Cells. Cells were treated with 0.1% DMSO or indicated concentrations of VR12684 for 12 h. Total cell lysates were prepared, and 50 μg of protein were subjected to SDS-PAGE followed by Western blot analysis and chemiluminescence detection. (a) Representative protein bands of KKU-M156 cells. (b) Histograms showing density of target protein normalized to β-actin protein of KKU-M156 cells. Each value represents mean ± SE of three independent experiments. *p<0.05, **p<0.01, ***p<0.001 versus the 0.1% DMSO-treated cells.

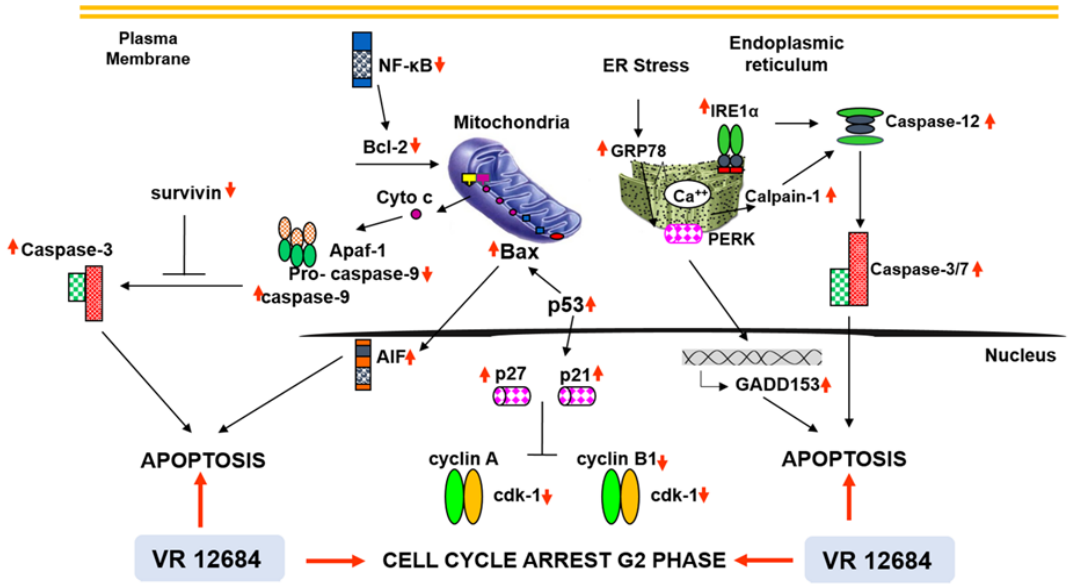

Figure 5. Possible Signaling Pathway in VR12684-Induced Cell Cycle Arrest and Apoptosis of Cholangiocarcinoma Cells. Upward and downward arrows indicate the upregulated and downregulated proteins in VR12684 treated CCA cells.

al., 2001). Sustained production of the cleaved form of caspase-7 was evident after VR12684 treatment, with a concomitant decrease in procaspase-7. Caspase-12 activation was assessed by an increase in the level of its cleaved form over the basal level in untreated cells. Since caspase-12 activation is responsible for ER stress-mediated apoptosis, these data indicated that VR12684 elicited apoptosis through ER stress in KKU-M156 cells. To further define the Ca^{2+}-mediated signaling pathway, we examined whether calpain-1 was activated by VR12684 treatment. As shown in Figure 4a and b, VR12684 significantly decreased the protein level of calpain-1, while significantly increasing the protein level of its cleaved form in a dose-dependent manner.

Discussion

Our results suggested that VR12684 promoted strong growth inhibitory activity against the KKU-M156 cell line. VR12684 induced KKU-M156 cell arrest in the G2 phase through a decrease in cyclin B1 and Cdk1 protein expressions while increasing p53, p21 and p27 protein expressions. KKU-M156 cell death induced by VR12684 in turn induced mitochondria-mediated apoptosis by increasing the Bax and AIF, and decreasing Bcl-2 and survivin and then subsequent activation of caspase-9 and -3. In addition, VR12684 could also induce apoptosis through the ER stress-mediated pathway by up-regulation of GRP78, IRE1α and GADD153 levels leading to down-regulation of Bcl-2 and activation of calpain, caspase-7 and -12. The underlying mechanism of VR12684 is summarized in Figure 5.

Early during the G2 phase, cyclin A was activated and formed a complex with Cdk1. In the late state, cyclin B1 was accumulated and formed a kinase complex with Cdk1. The cyclin A/Cdk2 complex plays a key role during S phase progression and the cyclin B1/Cdk1 complex controls cell entry and progression of the mitotic phase (M phase) (Coqueret et al., 2012). Cdk1 is a cell-cycle kinase responsible for the regulation of G2 progression and G2/M transition in all eukaryotic cells (Donzelli et al., 2003). Western blot analysis of cell cycle regulators in VR12684-treated KKU-M156 cells showed that protein expressions of cyclin A slightly decreased, whereas for cyclin B1 and Cdk1 there were significant dose-dependent decreases. The upstream regulators of the cyclin/Cdk complexes were also analyzed; we found that VR12684 peptide induced up regulation of p53, p21 and p27 protein expressions in this cell line. Further morphological examination revealed that VR12684-treated KKU-M156 cells showed G2 arrest. Our results support previous study in which the 7-mer cyclic peptide was reported to induce down-regulations of G2-specific cyclins (cyclin A, B1) in MCF-7cells (Ubol et al., 2007).

The ER is the site for the synthesis and folding of secreted and membrane-bound protein, and calcium storage. When protein folding in the ER is disrupted by alterations in homeostasis in the ER lumen, the unfolded protein response (UPR) will be activated. UPR is a self-protective mechanism, aimed at restoring normal ER function. To date there are three distinct transmembrane stress sensors in the UPR consisting of PERK, ATF6 and IRE1 (Bernales et al., 2006). Normally, in the resting state, these sensors associate with GRP78/Bip separately. When ER homeostasis is perturbed, GRP78 is released from these sensors and binds to unfolded/misfolded proteins that accumulate in the ER lumen, resulting in activated PERK, ATF6 and IRE1 triggering the UPR (Bertolotti et al., 2000; Liu et al., 2003; Ma et al., 2002; Shen et al., 2002). Our data were consistent with previous studies in human mammary epithelial (MCF10A) cells in which capsaicin induced apoptosis through ER stress-mediated pathways (Lee et al., 2009).

CHOP (CEBP homologous protein) is belonging

to the CCAAT/enhancer-binding protein (C/EBP) family, also known as DNA damage-inducible protein 153 (GADD153). The GADD153 is a downstream transcriptional target of ATF6 and PERK/eIF2α/ATF4. CHOP is a transcription factor that down-regulates the expression of Bcl-2 and thereby is proposed to promote apoptosis (Wang et al., 1996). In response to ER stress, IRE1α has been found to interact with c-Jun NH2-terminal inhibitory kinase and to recruit the adaptor protein TNF receptor-associated factor 2 (TRAF2) to the ER membrane. The recruitment of TRAF2 to IRE1α also permits TRAF2 to dissociate from procaspase-12 residing on the cytoplasmic side of the ER membrane, which is required for the activation of procaspase-12 (Yoneda et al., 2001). Under resting conditions, the pro-apoptotic BAX and BAK (BAX/BAK) are kept inactive by interaction with Bcl-2 both on the mitochondrial and ER membranes. ER stress leads to induction of the CHOP protein which will down-regulate the expression of Bcl-2 (Wu et al., 2016). BAK and BAX then undergo conformational alteration in the ER membrane to permit Ca^{2+} efflux, which activates the calcium-dependent protease m-calpain in the cytoplasm and subsequently cleaves and activates ER-resident procaspase-12 and leads to activation of the caspase cascade (Szegezdi et al., 2006). In addition, caspase-7, which translocate from the cytosol to the cytoplasmic side of the ER membrane in response to ER stress, will interact with and cleave caspase-12, leading to its activation. Previously, capsaicin has been reported to induce ER stress-mediated apoptosis pathways by down-regulation of Bcl-2 expression in human mammary epithelial (MCF10A) cells (Lee et al., 2009). In normal, human bronchial, polarized epithelial cells, Tg (T9033) has been reported to induce ER stress-mediated apoptosis through calpain activation and consequent activation of caspase-12 (Kerbiriou et al., 2009). VR-3848 has been reported to induce GADD153 and caspase-12 expression in a breast cancer (MCF-7) cell line (Ubol et al., 2007). Consistent with the previous studies, we found that VR12684 significantly decreased the protein expression of Bcl-2, calpain-1, pro-caspase-7 and pro-caspase-12 while significantly increasing Bax, GADD153, cleaved calpain-1, activated caspase-7 and the -12 in KKU-M156 cell line. These results suggested that VR12684 induced apoptosis in KKU-M156 cells through the ER stress-mediated apoptosis pathway.

In conclusion, VR12684 inhibited KKU-M156 cells mediated through cell cycle arrest and the induction of apoptosis. The growth inhibitory activities of VR12684 in KKU-M156 cells were due to cell cycle arrest in the G2 phase by altering the regulation of Cdk and p53 expression, resulting in cell arrest. The proposed apoptosis pathways for this study were mitochondria- and endoplasmic reticulum-associated intrinsic pathways. The present findings perhaps suggest that VR12684 has strong potential for development as a therapeutic agent against human cancers. However, further molecular mechanism studies are required to assess the effects of these two compounds in in vivo models of CCA cancer.

Author Contribution Statement

CH designed experiment. WP performed the experiments. VR separated VR12684. TB performed data analysis. PB analyzed the data, wrote and reviewed the manuscript. All authors read and approved the final manuscript.

Acknowledgements

The authors acknowledge the Kasetsart University Research and Development Institute (KURDI), Bangkok, Thailand for assistance with English editing.

Funding Statement

The present study was supported in part by grants from the Center of Excellence for Innovation in Chemistry, Commission on Higher Education, Bangkok and the Research Administration and Academic Service Division, Kasetsart University Chalermphrakiat Sakon Nakhon Province Campus, Sakon Nakhon, Thailand.

Data availability

The data that support the findings of this study are available from the corresponding author on request.

Conflict of interest

The authors declare that they have no competing interests.

References

Banales JM, Cardinale V, Carpino G, et al (2016). Expert consensus document: Cholangiocarcinoma: current knowledge and future perspectives consensus statement from the European Network for the Study of Cholangiocarcinoma (ENS-CCA). *Nat Rev Gastroenterol Hepatol*, **13**, 261-80.

Bernales S, Papa FR, Walter P (2006). Intracellular signaling by the unfolded protein response. *Annu Rev Cell Dev Biol*, **22**, 487-508.

Bertolotti A, Zhang Y, Hendershot LM, Harding HP, Ron D (2000). Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. *Nat Cell Biol*, **2**, 326-32.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, **72**, 248-54.

Bridgewater J, Galle PR, Khan SA, et al (2014). Guidelines for the diagnosis and management of intrahepatic cholangiocarcinoma. *J Hepatol*, **60**, 1268-89.

Coqueret O (2002). Linking cyclins to transcriptional control. *Gene*, **299**, 35-55.

Donzelli M, Draetta GF (2003). Regulating mammalian checkpoints through Cdc25 inactivation. *EMBO Rep*, **4**, 671-7.

Hahnvajanawong C, Wattanawongdon W, Chomvarin C, et al (2014). Synergistic effects of isomorellin and forbesione with doxorubicin on apoptosis induction in human cholangiocarcinoma cell lines. *Cancer Cell Int*, **14**, 68.

Intahphuak S, Panthong A, Kanjanapothi D, et al (2004). Anti-inflammatory and analgesic activities of *Mallotus spodocarpus* Airy shaw. *J Ethnopharmacol*, **90**, 69-72.

Kerbiriou M, Teng L, Benz N, Trouve P, Ferec C (2009). The calpain, caspase 12, caspase 3 cascade leading to apoptosis is altered in F508del-CFTR expressing cells. *PLoS One*,

4, e8436.
Lee MJ, Kee KH, Suh CH, Lim SC, Oh SH (2009). Capsaicin-induced apoptosis is regulated by endoplasmic reticulum stress- and calpain-mediated mitochondrial cell death pathways. *Toxicology*, **264**, 205-14.
Liu CY, Xu Z, Kaufman RJ (2003). Structure and intermolecular interactions of the luminal dimerization domain of human IRE1alpha. *J Biol Chem*, **278**, 17680-7.
Loaharanu P, Sornmani S (1991). Preliminary estimates of economic impact of liver fluke infection in Thailand and the feasibility of irradiation as a control measure. *Southeast Asian J Trop Med Public Health*, **22**, 384-90.
Ma K, Vattem KM, Wek RC (2002). Dimerization and release of molecular chaperone inhibition facilitate activation of eukaryotic initiation factor-2 kinase in response to endoplasmic reticulum stress. *J Biol Chem*, **277**, 18728-35.
Rao RV, Hermel E, Castro-Obregon S, et al (2001). Coupling endoplasmic reticulum stress to the cell death program. Mechanism of caspase activation. *J Biol Chem*, **276**, 33869-74.
Shen J, Chen X, Hendershot L, Prywes R (2002). ER stress regulation of ATF6 localization by dissociation of BiP/GRP78 binding and unmasking of Golgi localization signals. *Dev Cell*, **3**, 99-111.
Sripa B, Brindley PJ, Mulvenna J, et al (2012). The tumorigenic liver fluke Opisthorchis viverrini-multiple pathways to cancer. *Trends Parasitol*, **28**, 395-407.
Song S, Tan J, Miao Y, Zhang Q (2018). Crosstalk of ER stress-mediated autophagy and ER-phagy: Involvement of UPR and the core autophagy machinery. *J Cell Physiol*, **233**, 3867-74.
Szegezdi E, Logue SE, Gorman AM, Samali A (2006). Mediators of endoplasmic reticulum stress- induced apoptosis. *EMBO Rep*, **7**, 880-5.
Pootrakronchai R (2000). Apoptosis induction by VR3848 isolated from Euphorbiaceae in a human lung cancer cell line, Master of Science thesis (Toxicology), Mahidol University, Bangkok, Thailand.
Ubol S, Kramyu J, Masrinoul P, et al (2007). A novel cycloheptapeptide exerts strong anticancer activity via stimulation of multiple apoptotic pathways in caspase-3 deficient cancer cells. *Anticancer Res*, **27**, 2473-9.
Uthaisang W, Reutrakul V, Krachangchaeng C, Wilairat P, Fadeel B (2004). VR-3848, a novel peptide derived from Euphobiaceae, induces mitochondria-dependent apoptosis in human leukemia cells. *Cancer Lett*, **208**, 171-8.
Wang XZ, Lawson B, Brewer JW, et al (1996). Signals from the stressed endoplasmic reticulum induce C/EBP-homologous protein (CHOP/GADD153). *Mol Cell Biol*, **16**, 4273-80.
Wattanawongdon W, Hahnvajanawong C, Namwat N, et al (2015). Establishment and characterization of gemcitabine-resistant human cholangiocarcinoma cell lines with multidrug resistance and enhanced invasiveness. *Int J Oncol*, **47**, 398-410.
Woo JH, Kim YH, Choi YJ, et al (2003). Molecular mechanisms of curcumin-induced cytotoxicity: induction of apoptosis through generation of reactive oxygen species, down-regulation of Bcl-XL and IAP, the release of cytochrome c and inhibition of Akt. *Carcinogenesis*, **24**, 1199-208.
Wu J, Kaufman RJ (2006). From acute ER stress to physiological roles of the Unfolded Protein Response. *Cell Death Differ*, **13**, 374-84.
Xu B, Shan M, Wang F, et al (2013). Endoplasmic reticulum stress signaling involvement in manganese- induced nerve cell damage in organotypic brain slice cultures. *Toxicol Lett*, **222**, 239-46.
Yoneda T, Imaizumi K, Oono K, et al (2001). Activation of caspase-12, an endoplastic reticulum (ER) resident caspase, through tumor necrosis factor receptor-associated factor 2-dependent mechanism in response to the ER stress. *J Biol Chem*, **276**, 13935-40.