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European Journal of Pharmacology 503 (2004) 17–24

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Investigation of extrinsic and intrinsic apoptosis pathways of new clerodane diterpenoids in human prostate cancer PC-3 cells

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Received 6 May 2004; received in revised form 7 September 2004; accepted 10 September 2004
Available online 3 October 2004

Abstract

In our continuing search to discover bioactive compounds from natural products, we isolated six new clerodane diterpenes, caseamembrins A to F, from *Casearia membranacea* and examined their antiproliferative activities in human hormone-resistant prostate cancer PC-3 cells. All of these compounds displayed effective antiproliferative activity using sulforhodamine B assays and induced cell apoptosis by a terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL)-reaction technique. The data demonstrated that caseamembrin C was the most effective compound among these clerodane diterpenoids. Caseamembrin C induced down-regulation of Bcl-2 and Bcl-xL expression, while up-regulation of proapoptotic protein Mcl-1S (short chain), suggesting that these Bcl-2 family member proteins may play a role on arbitrating the apoptotic cell death. Caseamembrin C also induced the up-regulation of Fas ligand (FasL) expression, cleavage and activation of caspase-8 and caspase-9, Bid cleavage and activation of executor caspase-3. However, z-IETD-FMK (Z-Ile-Glu-Thr-Asp-fluoromethyl ketone, a selective caspase-8 inhibitor) almost completely inhibited caseamembrin C-induced Bid cleavage without any modification of caspase-9 activation, indicating that the extrinsic pathway of FasL/caspase-8/Bid cascade only played a minor role in the apoptotic signaling. Taken together, it is suggested that caseamembrin C-induced apoptosis is predominantly through the activation of intrinsic apoptosis pathways by causing the down-regulation of Bcl-2 and Bcl-xL expression, up-regulation of Mcl-1S protein and activation of caspase-9 and caspase-3.

Keywords: Clerodane diterpene; *Casearia membranacea*; Caseamembrin; Bcl-2 family member protein; Caspase

1. Introduction

Prostate cancer is the most common lethal form of malignancy in North American men and one of the leading causes of cancer death in men in Taiwan. In recent years, the mortality of prostate cancer has been increasing; it is near six in every 100,000 people and responsible for about 1000 deaths annually in Taiwan. Most prostate cancers are generally slow-growing malignancies and present themselves as mixtures of androgen-dependent and androgen-independent cells during the clinical diagnosis (Tang and Porter, 1997; Russell et al., 1998). Age and hormone are two known factors influencing the incidence of prostate cancer and, because of that, cancer cells initially respond to androgen withdrawal by undergoing apoptosis among the hormone-dependent population. However, patients with advanced or metastatic prostate cancer develop hormone-refractory status that becomes fatal because of the growth of androgen-independent tumor cells and the emergence of tumor clones. Therefore, the potential cancer chemotherapy to cause apoptosis in metastatic prostate cancer is necessary and urgent for the clinical treatment.

It has been well evident that there are two major apoptosis pathways, including intrinsic and extrinsic

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doi:10.1016/j.ejphar.2004.09.040

apoptosis signaling, in cells responsive to apoptotic stimuli (Chen and Wang, 2002; Budihardjo et al., 1999). The intrinsic apoptosis pathway is a mitochondria-involved signaling, and caspase-9 has been suggested as the predominant initiator caspase in this mitochondrial pathway. In the presence of ATP, the association of procaspase-9 with cytochrome *c* and the adaptor molecule apoptotic protease-activating factor 1 (Apaf-1) and oligomerization of this complex cause the activation of caspase-9 (Chen and Wang, 2002; Budihardjo et al., 1999; Cain et al., 2002). In contrast, the extrinsic apoptosis pathway is mediated by death receptors, such as the receptors for Fas and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), and caspase-8 is a major initiator caspase in this pathway (Petak and Houghton, 2001; Scaffidi et al., 1998). Recently, it has been suggested that there is cross-talk between these two apoptosis pathways. Bid, a proapoptotic member of Bcl-2 family, can be cleaved by caspase-8 to form truncated Bid (tBid). The translocation of tBid to mitochondria leads to the release of cytochrome *c* into cytosol and the formation of the apoptosome with procaspase-9 and Apaf-1 (Petak and Houghton, 2001; Scaffidi et al., 1998; Sun et al., 1999). The activation of caspase-9 in the apoptosome subsequently triggers the activation of downstream effectors, such as caspase-3. The activated caspase-3 can contribute a recruitment amplification to activate caspase-8 (Slee et al., 1999).

The clerodane diterpenoids constitute a large class of natural products. The number of natural clerodane diterpenoids has grown rapidly in recent years (Bruno et al., 2002), and many of them, especially those that are highly oxygenated, display effective insect antifeedant, antibacterial and antifungal properties (Merritt and Ley, 1992). Recently, the antitumor properties of clerodane diterpenoids have been investigated (Sai Prakash et al., 2002; Grynberg et al., 1999; Oberlies et al., 2002). However, there is little research elucidating the antitumor mechanism of the clerodane diterpenoids although the antitumor efficacy has been examined. In our continuing search to discover bioactive compounds from natural products, we isolated six new clerodane diterpenes, caseamembrins A–F, from *Casearia membranacea* and examined their antiproliferative activities in human hormone-resistant prostate cancer PC-3 cells, using sulforhodamine B assay method, a standard screening test in National Cancer Institute in USA. We found that all of these compounds displayed effective anticancer activities (Shen et al., 2004). In the present work, the caseamembrin-induced apoptotic mechanism was elucidated, and both the intrinsic and extrinsic apoptosis pathways were examined. We investigated the alteration in the expression of Bcl-2 family of proteins, as well as the cleavage and activation of several initiator and effector caspases in response to caseamembrin stimulus. To our knowledge, this is the first report that both intrinsic and extrinsic apoptosis pathways are investigated in clerodane diterpene-induced apoptosis in human cancer cells.

2. Materials and methods

2.1. Materials

RPMI-1640 medium, fetal bovine serum, penicillin, streptomycin and all other tissue culture reagents were obtained from GIBCO/BRL Life Technologies (Grand Island, NY, USA). EGTA, EDTA (disodium salt), leupeptin, dithiothreitol, phenylmethylsulfonyl fluoride, sulforhodamine B, aprotinin, β-isopropanol and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). Terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) apoptosis detection kits were from Promega (Madison, WI, USA). Antibodies to Mcl-1L/1S, Fas ligand (FasL), were from Santa Cruz (Delaware Ave., USA). Antibodies to Bid, caspase-8 and caspase-9 were from Cell Signaling (Beverly, MA, USA). Polycolonal anti-Bcl-xL antibody was from Upstate. Antibodies to Bcl-2, Bax, Fas and horseradish peroxidase-conjugated antimouse and horseradish peroxidase-conjugated antirabbit antibodies were from Transduction Lab (Lexington, KY, USA). Monoclonal antibody to caspase-3 was from IMGENEX (San Diego, CA, USA). Caspase-8 inhibitor, Z-Ile-Glu-Thr-Asp-fluoromethyl ketone (z-IETD-FMK), was from R&D Systems (Minneapolis, MN, USA). Clerodane diterpenes, caseamembrin A to F (Fig. 1), were isolated from the leaves and twigs of *C. membranacea*, and their structures were established on the basis of extensive 1D and 2D nuclear magnetic resonance (NMR) spectroscopic analysis (Shen

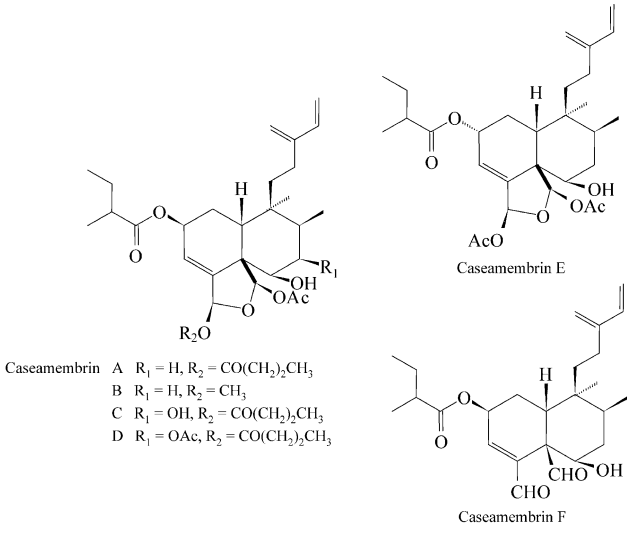

Fig. 1. The chemical structures of caseamembrin A to F.

et al., 2004). The purities of these compounds are more than 98%, which was identified and examined by NMR, mass and high-performance liquid chromatography (HPLC) detections.

2.2. Cell cultures

PC-3 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (v/v) and penicillin (100 units/ml)/streptomycin (100 μg/ml). Cultures were maintained in a humidified incubator at 37 °C in 5% CO_2/95% air.

2.3. Sulforhodamine B assays

Cells were inoculated into 96-well microtiter plates in RPMI-1640 medium containing 5% fetal bovine serum and maintained in a humidified incubator at 37 °C in 5% CO_2/95% air. After 24 h, two plates of each cell line were fixed in situ with trichloroacetic acid to represent a measurement at the time of drug addition (T_0). Additionally, vehicle or caseamembrin A to F was added to the cells of the other plates and incubated for an additional 48 h. The assay was terminated by the addition of cold trichloroacetic acid. After three-time washout with tap water, sulforhodamine B solution at 0.4% (w/v) in 1% acetic acid was added to each well, and plates were incubated for 10 min at room temperature. The unbound dye was removed by three-time washing with 1% acetic acid, and the plates were air dried. Bound sulforhodamine B was subsequently solubilized with 10 mM of trizma base, and the absorbance was read at a wavelength of 515 nm. Using the following absorbance measurements, such as time zero (T_0), control growth (C) and cell growth in the presence of compounds (T_x), the percentage growth was calculated at each of the compound concentrations levels. Percentage growth inhibition was calculated as $[(T_x-T_0)/(C-T_0)]\times 100$ for concentrations for which $T_x \geq T_0$.

2.4. In situ labeling of apoptotic cells

In situ detection of apoptotic cells was carried out using TUNEL apoptosis detection methods. The terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) identifies apoptotic cells in situ, using TdT to transfer biotin-dUTP to the free 3′ -OH of cleaved DNA. The biotin-labeled cleavage sites were then visualized by reaction with fluorescein-conjugated avidin (avidin–fluorescein isothiocyanate). Cells were cultured in chamber slides for 24 h and then treated with caseamembrin C for another 24 h. After the incubation period, cells were washed twice with phosphate-buffered saline (137 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4), fixed for 1 min with ice-cold ethanol/acetic acid (1:1) solution and then washed three times with phosphate-buffered saline. The fixed cells were permeabilized in ice-cold 0.2% triton ×−100 detergent for 5 min and then washed three times with phosphate-buffered saline. Staining was carried out according to the protocol provided by the suppliers. Finally, the photomicrographs were obtained with a fluorescence microscope (Nikon).

2.5. Mitochondrial MTT reduction activity assay

Cells were incubated in the absence or presence of caseamembrin C for the indicated time courses, and then the mitochondrial MTT reduction activity in PC-3 cells was assessed. MTT was dissolved in phosphate-buffered saline at a concentration of 5 mg/ml and filtered. From the stock solution, 10 μl per 100 μl of medium was added to each well, and plates were gently shaken and incubated at 37 °C for 1 h. After the loading of MTT, the medium was replaced with 100 μl of acidified β-isopropanol and was left for 5 to 10 min at room temperature for color development, and then the 96-well plate was read by enzyme-linked immunosorbent assay reader (570 nm) to get the absorbance density values.

2.6. Western blotting analysis

After the indicated exposure time of cells to vehicle or caseamembrin C, cells were washed twice with ice-cold phosphate-buffered saline, and reaction was terminated by the addition of 100 μl of ice-cold lysis buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1% triton ×−100). For Western blot analysis, the amount of proteins (40 μg) was separated through electrophoresis in a 10% or 15% polyacrylamide gel and transferred to a nitrocellulose membrane. After an overnight incubation at 4°C in phosphate-buffered saline/5% nonfat milk, the membrane was washed with phosphate-buffered saline/0.1% Tween 20 for 1 h and immunoreacted with the indicated antibody for 2 h at room temperature. After four washings with phosphate-buffered saline/0.1% Tween 20, the antimouse or anti-rabbit immunoglobulin (IgG; dilute 1:2000) was applied to the membranes for 1 h at room temperature. The membranes were washed with phosphate-buffered saline/0.1% Tween 20 for 1 h, and the detection of signal was performed with an enhanced chemiluminescence detection kit (Amersham).

2.7. Statistics and data analysis

Data are presented as the mean±S.E.M. for the indicated number of separate experiments. Statistical analysis of data was performed with one-way analysis of variance (ANOVA) followed by a t-test, and P-values less than 0.05 were considered significant.

3. Results

3.1. Effect of several caseamembrins on the inhibition of cell proliferation in PC-3 cells

The effect of several caseamembrins on the inhibition of cell proliferation was examined using sulforhodamine B assay to measure cellular protein content (Skehan et al., 1990). The data demonstrated that all of the caseamembrins (A to F) displayed the inhibitory effect of cell proliferation in a concentration-dependent manner, with IC_{50} values of 1.2, 24.2, 0.5, 1.4, 1.9 and 2.6 μM, respectively (Fig. 2) and total growth inhibition at 10.6, 94.5, 5.3, 7.1, 10.7 and 9.2 μM, respectively. The data indicated that caseamembrin C was the most effective compound among these clerodane diterpenoids. To identify the mode of cell death induced by caseamembrin C, we labeled the fragmented DNA of apoptotic cells by TUNEL reaction technique. The data showed that there was little positive staining in the vehicle control cells, while a strong positive labeling was detected in most of the cells in response to caseamembrin C. Under a high magnification (×1000), the fragmented and condensed DNA was detected in caseamembrin C-treated cells (Fig. 3), indicating that caseamembrin C is capable of inducing apoptotic cell death in PC-3 cells.

3.2. Effect of caseamembrin C on the expression of Bcl-2 family of proteins

The Bcl-2 family of proteins, including both inhibitors and promoters of apoptosis, is involved in the controlling of mitochondrial permeability, as well as regulation of caspase activation. In this study, several Bcl-2 family proteins, including antiapoptotic (Bcl-2, Bcl-xL and Mcl-1L) and proapoptotic members (Bax, Bad and Mcl-1S), were

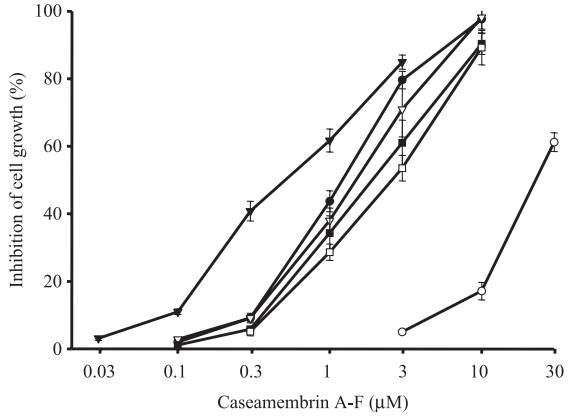

Fig. 2. Effect of several caseamembrins on the inhibition of PC-3 cell growth. Cells were treated with vehicle or the indicated concentration of caseamembrins in 5% fetal bovine serum for 48 h. Then, the cells were fixed and stained with sulforhodamine B, and the cell growth was detected and quantified as described in the Materials and methods section. Data are expressed as the mean±S.E.M. of five determinations (each in quadruplicate).

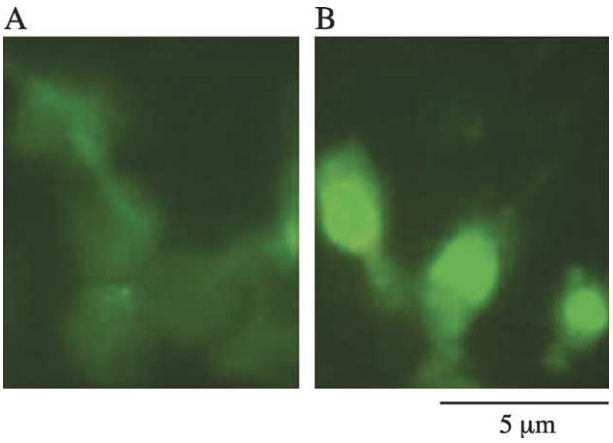

Fig. 3. Identification of caseamembrin C-induced apoptosis in PC-3 cells. Cells were cultured onto chamber slide and allowed to attach for 24 h. Cells were incubated in the absence (A) or presence of 3 μM caseamembrin C (B) for further 24 h. Then, the apoptotic effect was detected by TUNEL-reaction technique, as described in the Materials and methods section (1000× magnification).

detected in response to caseamembrin C in PC-3 cells. The data showed that the expression of proapoptotic protein Mcl-1S but not Bax and Bad was increased by caseamembrin C (Fig. 4). Whereas the expressions of the antiapoptotic members, Bcl-2 and Bcl-xL, were down-regulated by caseamembrin C in a concentration-dependent manner. The data suggest that Mcl-1S, Bcl-2 and Bcl-xL may play a role on arbitrating the caseamembrin C-induced apoptosis. It is worth noting that the expression of antiapoptotic protein, Mcl-1L, was moderately up-regulated by caseamembrin C with a concomitant increase of cleavage of this protein into 28 kDa cleavage product (Fig. 4). It seems contradictory to caseamembrin C-induced apoptotic

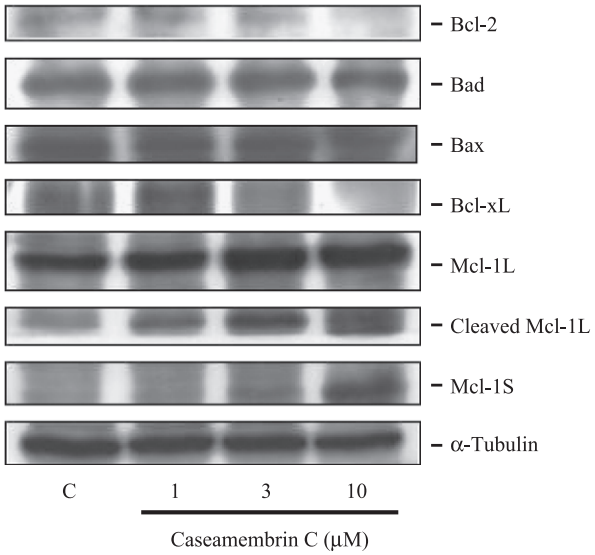

Fig. 4. Effect of caseamembrin C on the expression of Bcl-2 family of proteins. Cells were incubated in the absence (control) or presence of the indicated concentration of caseamembrin C for 16 h. Then the cells were harvested and lysed for the detection of protein expressions by Western blot analysis, as described in the Materials and methods section.

reaction. The reason will be discussed in the Discussion section below.

3.3. Effect of caseamembrin C on the activation of caspases

To determine which caspases were involved in caseamembrin C-induced apoptosis, the expressions of activated caspases were detected by Western blotting. In control cells, caspase-8, caspase-9 and caspase-3 were all present as uncleaved zymogens. However, following treatment with caseamembrin C, the cleavage of caspases to catalytically active fragments was clearly detected in a concentration-dependent manner (Fig. 5), suggesting the activation of these caspases.

3.4. Examination of the contribution of extrinsic and intrinsic apoptosis pathway

Inasmuch as caseamembrin C induced the activation of caspase-8 and caspase-9, two predominant initiator caspases in extrinsic and intrinsic apoptosis pathways, respectively (Chen and Wang, 2002; Budihardjo et al., 1999; Cain et al., 2002; Petak and Houghton, 2001; Scaffidi et al., 1998), several related molecules and substrates were examined to determine which pathway contributes to caseamembrin C-mediated apoptotic cell death. At first, the death receptor, Fas, and its ligand were detected and, the data showed that Fas was present in the control cells although there was little alteration of this protein expression in cells responsive to

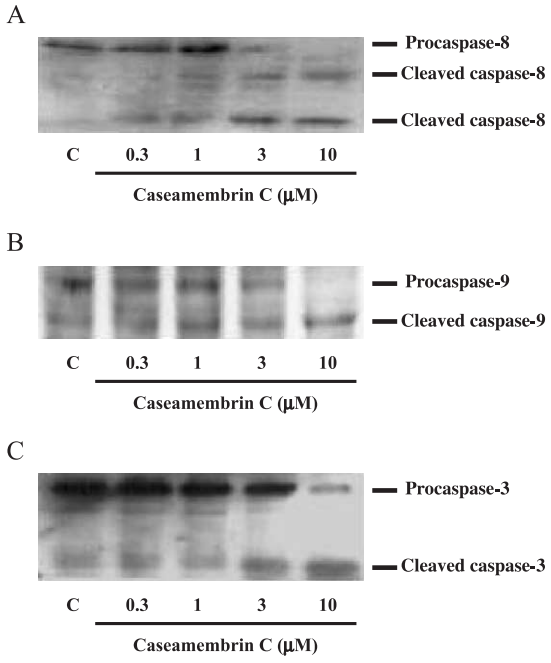

Fig. 5. Effect of caseamembrin C on the expression of several procaspases and cleaved caspases. Cells were incubated in the absence (control) or presence of the indicated concentration of caseamembrin C for 24 h. Then the cells were harvested and lysed for the detection of protein expressions by Western blot analysis, as described in the Materials and methods section.

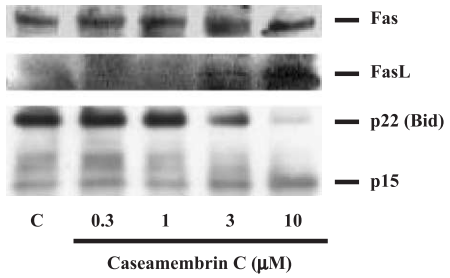

Fig. 6. Effect of caseamembrin C on the expression of Fas, FasL and Bid in PC-3 cells. Cells were incubated in the absence (control) or presence of the indicated concentration of caseamembrin C for 24 h. Then the cells were harvested and lysed for the detection of protein expressions by Western blot analysis, as described in the Materials and methods section.

caseamembrin C. With contrast, FasL was significantly up-regulated by caseamembrin C (Fig. 6). Additionally, the 22-kDa Bid, a proapoptotic member of Bcl-2 family, was also cleaved into a truncated (tBid) 15-kDa protein after the treatment of caseamembrin C (Fig. 6). The data revealed that caseamembrin C could activate an extrinsic pathway of FasL/caspase-8/Bid cascade.

There are several lines of evidence suggesting that caspase-9 is not only an initiator caspase of intrinsic apoptosis pathway but also a shared caspase upon the mitochondrial-dependent activation of extrinsic pathway (Petak and Houghton, 2001). To investigate whether caspase-9 activation was shared with both extrinsic and intrinsic pathways, a selective caspase-8 inhibitor, z-IETD-FMK, was used in this study to block caspase-8-mediated effect. As demonstrated in Fig. 7A, z-IETD-FMK (30 μM) almost entirely abolished caseamembrin C-induced cleavage of Bid, suggesting the complete blockade of caspase-8 activity. However, under similar treatment, z-IETD-FMK

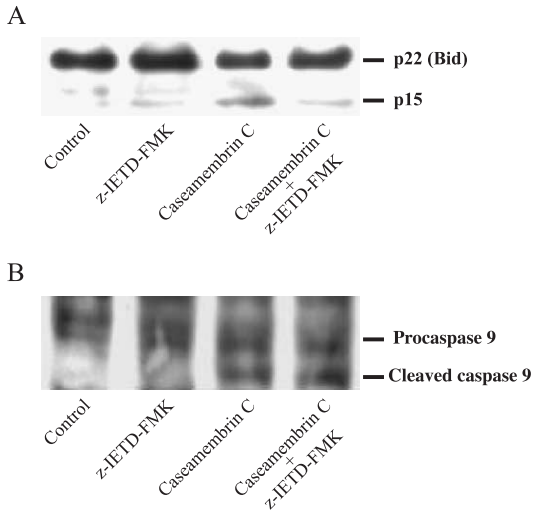

Fig. 7. Effect of z-IETD-FMK on caseamembrin C-induced Bid cleavage and caspase-9 activation. Cells were incubated in the absence (control) or presence of z-IETD-FMK (30 μM) for 30 min, then vehicle or caseamembrin C was added to the cells. After a 24-h incubation, cells were harvested and lysed for the detection of protein expressions by Western blot analysis, as described in the Materials and methods section.

could hardly modify caseamembrin C-induced caspase-9 activation (Fig. 7B). Additionally, it showed that z-IETD-FMK had little effect on caseamembrin C (3 μM)-induced inhibition of cell proliferation and cytotoxic effect by sulforhodamine B and mitochondrial MTT reduction assays, respectively (data not shown). These data indicate that the extrinsic pathway plays a minor role on caseamembrin C-induced effect. Furthermore, to further identify the involvement of caspase-9 and caspase-3, the enzymatic activities were also examined, and the data showed that caseamembrin C (10 μM) induced a significant increase of both caspase-9 and caspase-3 activities (3.22±0.68-fold and 4.91±0.40-fold, respectively).

4. Discussion

Clerodane diterpenoids have caused more and more attention in recent years for their widely biological activities, especially the cytotoxic effect on cancer cells (Sai Prakash et al., 2002; Grynberg et al., 1999; Oberlies et al., 2002). In the present work, we have purified several clerodane diterpenes from *C. membranacea* and examined their antiproliferative activities in PC-3 cells. The data showed that caseamembrin C was the most effective compound among these clerodane diterpenoids with an IC_{50} value of 0.5 μM. Furthermore, it showed that the antiproliferative potency of caseamembrin C was increased (IC_{50}=0.09 μM) in a 72-h treatment in PC-3 cells (data not shown). It seems that caseamembrin C displays potent antiproliferative activity among natural products and is worth further investigation of the action mechanism. At first, the caseamembrin C-induced apoptotic effect was identified with the detection of DNA fragmentation by strong positive TUNEL reaction. Apoptosis represents a general and delicately efficient cellular suicide pathway. In a lot of tumors, apoptosis is an important mechanism associated with the induction of tumor remission.

Two major targets are involved in the stimulating and progression of apoptotic cell death, one occurring in mitochondria and a second, the activation of caspase proteases (Arita et al., 2001). Bcl-2 family of proteins may contribute predominantly to the mitochondrial pathway of apoptosis in response to diverse cytotoxic agents. In mammals, Bcl-2 has more than 15 relatives, all of which share at least one conserved Bcl-2 homology (BH) domain. Bcl-2, Bcl-xL, Bcl-w, Mcl-1 and A1 are five antiapoptotic proteins in this family (Cory and Adams, 2002; Craig, 1995; Gibson et al., 1996). It has been well evident that Bcl-2 and its closest homologues, Bcl-xL and Bcl-w, potently inhibit apoptosis in response to numerous cytotoxic insults; whereas A1 and Mcl-1, two less well studied proteins, seem to have weaker survival activity (Cory and Adams, 2002). Bax and the BH3-only families are two groups of proteins that promote apoptosis. Members of Bax family have sequences similar to those in Bcl-2, especially in the BH1, BH2 and BH3 regions, while the other proapoptotic proteins have only the BH3 motif (Oltvai et al., 1993; Bouillet and Strasser, 2002). During apoptosis, Bax family proteins oligomerize in the mitochondrial outer membrane and disrupt its integrity, inducing the release of cytochrome *c* and subsequent activation of caspase-9 (Cain et al., 2002; Cory and Adams, 2002; Oltvai et al., 1993). The BH3-only proteins act as direct antagonists and inactivate the prosurvival members of Bcl-2 family (Bouillet and Strasser, 2002). Additionally, a short splicing variant of the Mcl-1 mRNA encoding a protein, termed Mcl-1 short (Mcl-1S) as compared with the full-length Mcl-1 (Mcl-1L), has been identified recently (Bae et al., 2000). It has been suggested that overexpression of Mcl-1S induces cell apoptosis. Therefore, the Mcl-1S variant represents a new proapoptotic protein (Bae et al., 2000). Three antiapoptotic proteins including Bcl-2, Bcl-xL and Mcl-1L, which were expressed more abundantly than A1 and Bcl-w in PC-3 cells, were investigated in this study. The data showed that the expressions of both Bcl-2 and Bcl-xL were down-regulated by caseamembrin C in a concentration-dependent manner. Furthermore, among three proapoptotic proteins tested in this study, only the expression of Mcl-1S was profoundly increased by caseamembrin C, suggesting that Bcl-2, Bcl-xL and Mcl-1S may play a central role on the apoptotic signaling pathway. However, it is worth noting that the expression of the antiapoptotic protein Mcl-1L was moderately up-regulated by caseamembrin C with a concomitant increase of cleavage of this protein into about 28-kDa cleavage product. The cleavage of Mcl-1L that can attenuate its antiapoptotic effect may be executed by caspase-3 inasmuch as it has been suggested that both caspase-3 and caspase-7 are able to process Mcl-1L to give a major 28-kDa product, while caspase-3 is much more effective than caspase-7 (Snowden et al., 2003). We suggest that caspase-3 activated by caseamembrin C (will be discussed below) could be the major caspase responsible for Mcl-1L cleavage, but we cannot exclude a possible contribution from caspase-7. However, one question would be raised, that the up-regulation of Mcl-1L was still detected although a concomitant cleavage occurred. One possible explanation is that the antiapoptotic effect of Mcl-1L is antagonized by Mcl-1S inasmuch as it has been suggested that Mcl-1S is capable of dimerizing with Mcl-1L and antagonizing Mcl-1L action (Bae et al., 2000). In this study, the expression of Mcl-1L was increased to 1.3-fold and 1.4-fold, while Mcl-1S to 1.6-fold and 3.1-fold, in response to 3 and 10 μM caseamembrin C, respectively. The net increase was toward to the proapoptotic Mcl-1S. Taken together, the data indicate that the down-regulation of Bcl-2 and Bcl-xL and the up-regulation of Mcl-1S could be responsible for caseamembrin C-induced apoptosis in PC-3 cells.

The other central pathway involved in the process of apoptotic cell death is the activation of the caspase proteases. Caspases are cysteine proteases, which are synthesized primarily as inactive precursors and are

activated during apoptosis as well as other biological process (Thornberry and Lazebnik, 1998; Schwerk and Schulze-Osthoff, 2003). Caspases are divided into two major functional groups, initiator caspases and effector caspases. Initiator caspases undergoing autocatalysis or activation via the formation of apoptosome are able to cleave and activate effector caspases and carry out the process of apoptotic cell death. Caspase-8 and caspase-9 are two major initiator caspases in extrinsic and intrinsic apoptosis pathways, respectively (Chen and Wang, 2002; Budihardjo et al., 1999). In this study, caseamembrin C induced the cleavage and activation of the initiator caspases, caspase-8 and caspase-9, and the executor caspase-3 in a concentration-dependent manner, indicating the involvement of both extrinsic and intrinsic apoptosis pathways. However, it has been suggested that the activation of caspase-8 causes the cleavage of Bid that in turn induces a subsequent activation of caspase-9 (Petak and Houghton, 2001; Scaffidi et al., 1998; Sun et al., 1999). The data in this study demonstrated that caseamembrin C significantly induced the cleavage of Bid into a 15-kDa fragment. Furthermore, caseamembrin C also induced a concentration-dependent increase of FasL expression. It is apparent that caseamembrin C induces an extrinsic apoptosis pathway in PC-3 cells. Besides, it raises one question that the activation of caspase-9 resulted from the intrinsic apoptosis pathway or partly through caspase-8/Bid signaling cascade. To clarify this question, a specific caspase-8 inhibitor, z-IETD-FMK, was used in this study. The data showed that z-IETD-FMK almost completely inhibited caseamembrin C-induced cleavage of Bid but had little effect on that of caspase-9 activation, indicating that the extrinsic apoptosis pathway mediated by FasL/caspase-8/Bid cascade only played a minor role on caspase-9 activation. Furthermore, z-IETD-FMK also showed little inhibitory effect on caseamembrin C-induced functional antiproliferative effect by both mitochondrial MTT reduction and sulforhodamine B assays. However, it is interesting and worth noting that extrinsic apoptosis pathway is activated while plays a minor role on caseamembrin C-mediated apoptotic effect. It has been suggested that there are two distinct signaling pathways in Fas-mediated apoptosis (Petak and Houghton, 2001; Sun et al., 1999; Kuwana et al., 1998). In type I cells, ligation and activation of Fas lead to strong activation of caspase-8 and caspase-3 without the involvement of mitochondria. In type II cells, the activation of caspase-8 is able to cleave the cytosolic substrate Bid and cause a subsequent activation of caspase-9. The caseamembrin C-induced effect in PC-3 cells can be classified as type II cells, and the activation of a small amount of caspase-8 is supposed to be less than type I cells, although there is no comparison with that of type I cells in this study. It could partly explain the minor contribution of caspase-8 on caseamembrin C-induced effect. However, it needs further investigation to make clear the fundamental role of caspase-8 in this study.

Conventional cancer chemotherapeutic drugs indirectly induce cancer cell apoptosis, but more effective outcomes should be reached by direct activation of the apoptotic machinery. Accordingly, approaches of anticancer agents that cause down-regulation of antiapoptotic Bcl-2 family of proteins and/or up-regulation of proapoptotic Bcl-2 family of proteins should be carried out. It is suggested that caseamembrins C displays effective antiproliferative activity in PC-3 cells. The intrinsic apoptosis pathway, including down-regulation of Bcl-2 and Bcl-xL, up-regulation of Mcl-1S and activation of initiator caspase-9, as well as executor caspase-3, may predominantly explain caseamembrin C-mediated apoptotic mechanism.

Acknowledgments

This work was supported by a research grant of the National Science Council of the Republic of China (91-3112-P-002-004-Y and 92-2323-B-002-010) and Committee on Chinese Medicine and Pharmacy, Department of Health, the Republic of China (CCMP92-RD-114).

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