F16, A Fraction from *Eurycoma longifolia* Jack Extract, Induces Apoptosis *via* a Caspase-9-independent Manner in MCF-7 Cells

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Abstract. *F16 is a plant-derived pharmacologically active fraction extracted from Eurycoma longifolia Jack. Previously, we have reported that F16 inhibited the proliferation of MCF-7 human breast cancer cells by inducing apoptotic cell death while having some degree of cytoselectivity on a normal human breast cell line, MCF-10A. In this study, we attempted to further elucidate the mode of action of F16. We found that the intrinsic apoptotic pathway was invoked, with the reduction of Bcl-2 protein. Then, executioner caspase-7 was cleaved and activated in response to F16 treatment. Furthermore, apoptosis in the MCF-7 cells was accompanied by the specific proteolytic cleavage of poly(ADP-ribose) polymerase-1 (PARP-1). Surprisingly, caspase-9 and p53 were unchanged with F16 treatment. We believe that the F16-induced apoptosis in MCF-7 cells occurs independently of caspase-9 and p53. Taken together, these results suggest that F16 from E. longifolia exerts anti-proliferative action and growth inhibition on MCF-7 cells through apoptosis induction and that it may have anticancer properties.*

In light of the continuing search for effective anticancer agents, plants are increasingly being considered as a reservoir of anticancer drugs (1). In cancer therapy, the focus is on strategies that suppress tumor growth by activating the apoptotic program in the cell (2-4). This idea is supported by the fact that commonly used chemotherapeutic drugs are effective because they induce this form of cancer cell death (5-7). Most cancer development is directly related to repression of apoptosis resulting in uncontrolled cellular growth (8).

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Key Words: Antiproliferative effect, apoptosis, PARP, *Eurycoma longifolia*, MCF-7.

The process of apoptosis was first described in 1972 (9) but its physiological significance was not fully appreciated until years later (10). In contrast to necrosis, apoptosis is a highly ordered process in which unwanted or damaged cells are removed from the organism, and is mediated by specialized intracellular biochemical pathways involving mainly a family of intracellular polypeptides (11). One of the major proteins involved in this process is the tumor suppressor protein p53, which mediates both cell cycle arrest and apoptosis (12). Two different pathways mediating drug-induced apoptosis have been characterized: one requires the activation of cell surface receptors, whilst the other directly targets mitochondria (13). Both apoptotic signals seem to be typically accompanied by the activation of cysteine aspartate-specific proteases called caspases (14). The primary function of caspases is to cleave cellular substrates, which results in amplification of the apoptotic process and the stepwise dismantling of the cell (15). Whilst the former pathway induces caspase-8 activation, the mitochondrial pathway leads to the release of apoptogenic factors such as cytochrome *c*, which binds Apaf-1 and procaspase-9, inducing caspase-9 activation in the cytoplasm (16). Both pathways then activate the effectors caspase-3 and -7, which cleave a number of substrate proteins, including the poly(ADP-ribose) polymerase-1 protein (PARP-1) (17).

Chemotherapy is commonly applied in breast cancer, but its results are often discouraging because of chemoresistance conferred by *p53* mutation and its toxicity to normal cells. Therefore, a novel pharmaceutical compound needs to be developed. Recently, F16, a pharmacologically active fraction extracted from the plant *Eurycoma longifolia*, has been reported to possess significant anti-proliferative activity against a human breast cancer cell line, MCF-7 (IC_{50}=15.23±0.66 μg/ml) with a certain degree of cytoselectivity on a human normal breast cell line, MCF-10A (IC_{50}=66.31±0.47 μg/ml). Furthermore, F16-triggered apoptosis on the human breast cancer cell line, MCF-7, is

mediated *via* suppression of Bcl-2 oncoprotein expression rather than BAX (18). The precise cellular mechanisms of the action of F16 on cancer cells are not completely understood. Therefore, in this study we aimed to investigate the *in vitro* anti-proliferative potential of F16 and ascertain its exact mechanism of inducing apoptosis in MCF-7 cells. A better understanding of how *E. longifolia* phytochemicals inhibit proliferation of cancer cells will enable them to be used more effectively and perhaps synergistically with other existing chemotherapeutic agents.

Materials and Methods

Chemicals and reagents. Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), penicillin-streptomycin and fungizon were purchased from Gibco (Grand Island, USA). Trypsin, dimethyl sulfoxide (DMSO) and anti-β actin (Clone AC15) antibody were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Gentamycin was from Atlantic Laboratories Corp. Ltd. (Thailand). Anti-p53 (clone PAb 122), anti-caspase-9 (Clone B40), anti-caspase-7 (Clone B94-1), and anti-PARP-1 (Clone 4C10-5) antibodies were purchased from Pharmingen (USA). Renaissance Western blot Chemiluminescence reagent Plus was from PerkinElmer (Boston, USA). Bradford reagent was from Bio-Rad laboratories (USA).

Preparation of F16 from E. longifolia. F16 was prepared from the root of *E. longifolia* as described elsewhere (18).

Cell culture conditions. The MCF-7 human mammary carcinoma cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). MCF-7 cells were maintained in DMEM supplemented with 5% FCS and antibiotics at 37˚C in a humidified atmosphere containing 5% CO_2. For the experiments, cells were removed from the flasks using a 0.025% trypsin solution.

Protein extraction. MCF-7 cells grown to 70% confluence were treated with F16 at 15 μg/ml for durations of 0, 2, 4, 6, 12 and 24 hours. At the indicated times, the cells were scraped after incubation in extraction buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1% (v/v) Triton-X, 1 mM phenylmethylsulfonyl fluoride, 10 mM glycerophosphate, 1 mM Na_3VO_4, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, 20 μg/ml leupeptin, and 5 mM DTT) and put on ice for 45 minutes. Cells were then submitted to 3 freeze-thaw cycles and centrifuged at 10,000 rpm for 20 min at 4˚C to remove cellular debris. The supernatant extracts were quantified for protein using Bradford procedures.

Western blotting. Electrophoresis was performed on 12% SDS-PAGE using 20 μg of reduced protein per lane. Proteins were then transferred to polyvinyldenefluoride (PVDF) membranes (PolyScreen, NEN Life Sciences, USA). Membranes were dried, preblocked with 5% non-fat milk in phosphate-buffered saline and 0.1% Tween-20, then incubated with primary antibodies of anti-caspase-9, anti-caspase-7, anti-PARP-1 or anti-β actin at room temperature for 120 min. Antibody recognition was detected with secondary antibody anti-mouse IgG linked to horseradish peroxidase at room temperature for 60 min. The expression levels were normalized to actin expression. Antibody-bound proteins were detected by the Renaissance Western blot Chemiluminescence reagent Plus. Following exposure on a Kodax BIOMAX X-ray film, densitometry analysis was performed with a GS 670 Imaging Densitometer with the software Molecular Analyst (Bio Rad, Hercules, USA). Each Western blot shown is representative of at least three separate experiments.

Statistical analysis. All data were expressed as mean±standard deviation. The statistical differences were analyzed using Student's *t*-test. Values of $p<0.05$ were considered significant.

Results

P53 protein was maintained at a basal level throughout the duration of F16 treatment. We examined, by Western blot analysis, whether F16 has any effect on the expression of this protein. As shown in Figure 1, no increase in p53 protein expression was detected. This demonstrated that F16-induced apoptosis was probably not mediated by altering p53 levels.

Procaspase-9 is not processed in F16-treated cells. We sought to determine whether procaspase-9 is processed as expected in MCF-7 cells treated with F16. By SDS-PAGE and subsequent Western blot analysis with a caspase-9-specific antibody, it was found that F16 treatment did not lead to the activation of the initiator caspase-9. No detectable changes in the amount of procaspase-9 following F16 treatment (Figure 2) were observed. From immunoblotting, the observed band was the 46 kDa proform of caspase-9 and the active 35 kDa subunit could not be detected.

Involvement of caspase-7 in F16 induced apoptosis. In the absence of detectable caspase-3 due to a gene deletion in the MCF-7 genome (26), we analyzed caspase-7 processing as a possible substitute. From immunoblot analysis, untreated MCF-7 cells exhibited the 38 kDa proform of caspase-7. When MCF-7 cells were treated with 15 μg/ml F16, the observed zymogen of caspase-7 slowly diminished in the course of the experiment (Figure 3) although the active 17 kDa fragment was not detected. The disappearance of the procaspase-7 band reflects the processing of the zymogen to generate the active form of caspase-7, as has been interpreted in previous reports (19-20).

F16 induces activation of PARP-1 in MCF-7 cells. Measurement of PARP-1 cleavage was used as a direct measure of apoptosis (21). Figure 4 clearly shows that MCF-7 cells that are induced by F16 undergo apoptosis through the disappearance of the uncleaved PARP-1 (113 kDa), although its cleaved 89 kDa fragment was not detected. The disappearance of the 113 kDa PARP-1 band reflects the processing of the zymogen to generate the cleaved 89 kDa fragment, as has been interpreted in a previous report (22).

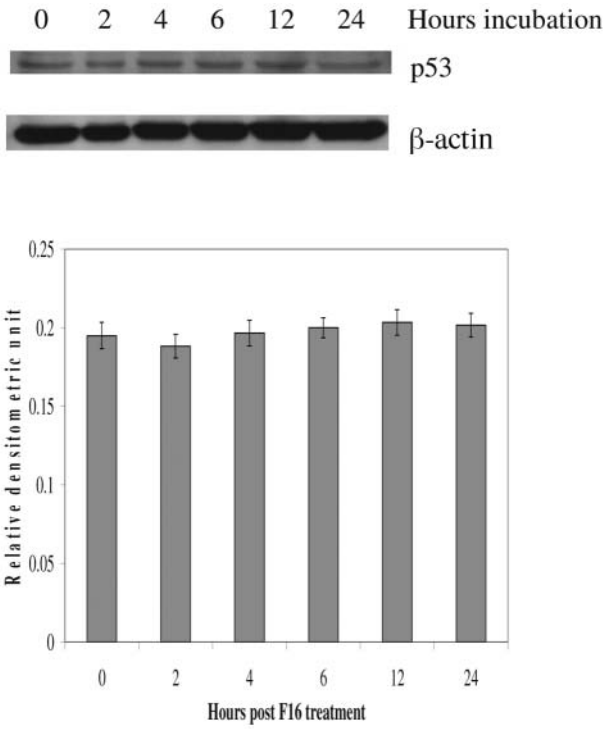

Figure 1. *Western blot analysis of p53 protein in F16-treated MCF-7 cells. MCF-7 cells treated with F16 for the indicated times were resolved on a 12% SDS-PAGE and submitted to Western blotting. P53 levels were not altered ($p>0.05$) and remained at the basal level throughout the experiment. Results are presented as means±SD of 6 independent experiments.*

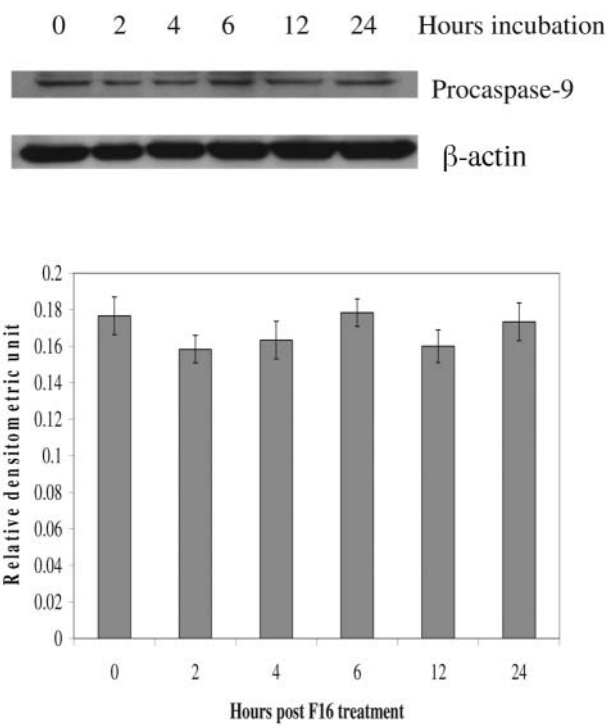

Figure 2. *Western blot analysis of procaspase-9 protein in F16-treated MCF-7 cells. MCF-7 cells treated with F16 for the indicated times were resolved on a 12% SDS-PAGE and submitted to Western blotting. Procaspase-9 levels were not altered ($p>0.05$) and remained at the basal level throughout the experiment. Results are presented as means±SD of 6 independent experiments.*

Discussion

In the present study, we provide evidence that a fraction of plant origin, F16, may be a promising new anticancer agent for human breast cancer. Previously, we have shown that MCF-7 cells proliferation was significantly inhibited by F16 (IC_{50}=15.23±0.66 μg/ml) and was shown to induce apoptosis in MCF-7 cells through the modulation of Bcl-2 protein levels (18). In addition to the loss of viability, F16 produces other typical manifestations leading to apoptosis, namely caspase activation. Here we found that PARP-1 was cleaved, following cleavage of caspase-7. Interestingly, our finding indicated that activation of caspase-7 was not through caspase-9. Neither the level of caspase-9 nor of p53 were altered in MCF-7 cells treated with F16.

p53 has been found to be importantly involved in apoptosis induced by a broad range of cytotoxic agents (23-24). However, after treatment with F16, immunoblotting results did not show any increase in p53 levels. This suggests that F16-induced apoptosis is probably not mediated by alteration of p53 levels. Although p53 has been found to play a critical role in apoptosis, this may not be the case for all cell lines or all types of DNA damage. Similarly, the action of drugs such as tamoxifen (25) and paclitaxel (26) were previously reported to be independent of p53.

In an effort to identify the underlying mechanisms of cancer, executioners of apoptosis such as caspases have been described. As caspase activation is a central requirement for apoptosis, elucidation of the caspase activation pathway invoked by cytotoxic agents may offer vital information in designing better treatment strategies. Introducing apoptosis in cancer cells is considered a valuable way for treatment of cancer (27). Until recently, the sole apical initiator in apoptosis pathways was assumed to be caspase-9, which is activated in a complex termed the apoptosome by the scaffold protein Apaf-1 and its cofactor cytochrome *c* (28-29). Caspase-9 is of particular interest because it initiates cell death in response to non-cytokine-mediated cellular stress, such as UV or chemical treatment (30). However, the apoptosome is not universally essential for Bcl-2-regulated apoptosis, because certain neuronal (31), hematopoietic and fibroblastoid cells (32) lacking Apaf-1 or caspase-9 readily undergo apoptosis in response to diverse insults. Here we found that F16-mediated apoptosis in MCF-7 cells was

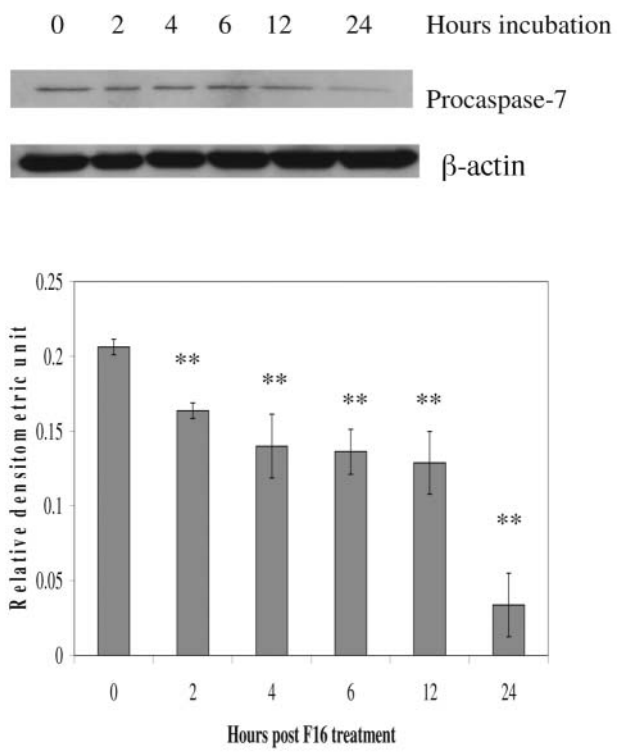

Figure 3. *Western blot analysis of procaspase-7 protein in F16-treated MCF-7 cells. MCF-7 cells treated with F16 for the indicated times were resolved on a 12% SDS-PAGE and submitted to Western blotting. Procaspase-7 protein expression decreased as early as 2 hours following F16 treatment. Results are presented as means±SD of 6 independent experiments. **p<0.005 statistically significant values relative to time 0.*

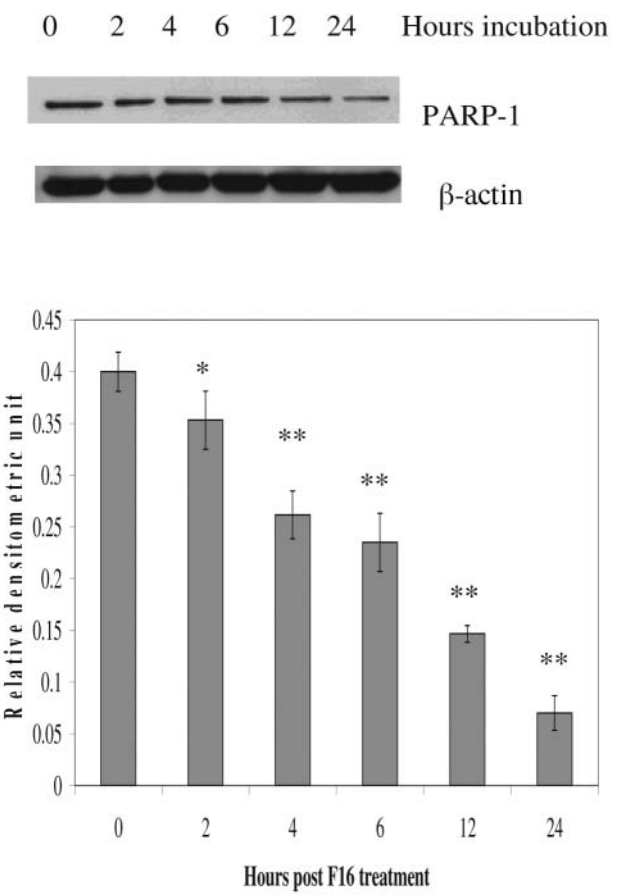

Figure 4. *Western blot analysis of PARP-1 protein in F16-treated MCF-7 cells. MCF-7 cells treated with F16 for the indicated times were resolved on a 12% SDS-PAGE and submitted to Western blotting. PARP-1 protein expression decreased as early as 2 hours following F16 treatment. Results are presented as means±SD of 6 independent experiments. *p<0.05, **p<0.005 statistically significant values relative to time 0.*

independent of caspase-9. As processing of this caspase did not occur, it is possible that the other initiator caspase may be involved in F16-induced apoptosis. Similarly, UV and taxol-induced apoptosis in MCF-7 cells are also mediated independently of caspase-9 (33-34).

MCF-7 cells are deficient of caspase-3 due to the deletion mutation in exon 3 of the gene (35). Studies using etoposide and doxorubicin, active chemotherapeutic agents and key adjuvant drugs for breast cancer treatment, concluded that MCF-7 cells were sensitized to apoptosis only when these cells were reconstituted with caspase-3 (36). Given the importance of caspase-3 in apoptotic execution, it is then postulated that caspase-3 deficiency might significantly contribute to chemotherapeutic resistance.

In our studies, we observed manifestations of apoptosis in F16-treated MCF-7 cells. Previous studies have also demonstrated similar apoptotic hallmarks in MCF-7 cells when induced with buprenorphine hydrochloride (37), UV (33), styrylpyrone derivative (38) and xanthorrhizol (39) respectively. These suggest that the mechanism for induction of apoptosis is present and functional in MCF-7 cells, but is dependent on external stimuli. Caspase-7 is highly related to caspase-3 and shows the same synthetic substrate specificity *in vitro* suggesting that caspase-3 and -7 possibly have overlapping roles in apoptosis (40). Without caspase-3, F16-treated MCF-7 cells may utilize an alternate caspase pathway to affect cell death. Here, we demonstrated that caspase-7 was activated in F16-induced apoptosis. Tumors accumulate mutations that increase their resistance to apoptotic inducers: *e.g.* abrogation of caspase-3 has been associated with acquired multidrug resistance (41). Therefore, finding new therapeutic agents that induce tumor cell apoptosis in a manner independent of caspase-3 may have important clinical implications (42). By not requiring caspase-3, F16 may evoke an apoptotic pathway different from that by clinical oncology drugs such as doxorubicin and etoposide, thus making it a promising agent for combination chemotherapy meriting further study.

PARP-1 was revealed as the downstream target in the F16-mediated mechanism, linking caspase-7 activation and nuclear apoptosis. The disappearance of the 113 kDa uncleaved PARP-1 band reflects the processing of the zymogen to generate the cleaved 89 kDa fragment, as has been interpreted in a previous report (22). These data support a previous study showing that caspase-7 catalytic activity can induce PARP-1 cleavage (43). PARP-1 inhibitors in combination with DNA damaging agents were found to increase DNA damage, thus suggesting the involvement of PARP-1 in cell death (44).

The results of this study collectively indicate that F16 contains component(s), possibly acting in concert, that inhibit the proliferation of the human breast cancer cell line MCF-7 by inducing apoptosis. F16 induced MCF-7 cell death by cleavage of caspase-7 and PARP-1 but independently of caspase-9 and p53. However, the upstream signaling of F16-induced apoptosis in MCF-7 cells remains elusive. Further study of the action of F16 on MCF-7 cells might open the door to a new approach for cancer therapy. The assumption that poly(ADP-ribosylation) plays a crucial role in carcinogenesis validates the growing interest in developing PARP-1 inhibitors to improve the efficacy of radio- and chemotherapy (44). Current studies to identify the component(s) responsible for the cytotoxic activity of F16 could yield a novel anticancer agent.

Acknowledgements

This work was supported by a grant from Massachusetts Institute of Technology (MAL-MIT 33-00-90173), USA. We thank Dr. N. Meenakshii for editing the manuscript.

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Received April 13, 2007
Revised July 24, 2007
Accepted August 1, 2007