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Protein Tyrosine Kinases, p56^{lck} and p59^{fyn}, and MAP Kinase JNK1 Provide an Early Signal Required for Upregulation of Fas Ligand Expression in Aburatubolactam C-Induced Apoptosis of Human Jurkat T Cells

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Abstract The signaling mechanism underlying aburatubolactam C-induced FasL upregulation was investigated in human Jurkat T cells. After treatment with aburatubolactam C, the src-family PTKs p56^{lck} and p59^{fyn}, and MAP kinases ERK2 and JNK1, were activated prior to FasL upregulation. Both p56^{lck} and p59^{fyn} were directly activated 2.4- and 2.2-fold, respectively, *in vitro* by aburatubolactam C. The aburatubolactam C-induced cellular changes, including the activation of ERK2 and JNK1, and FasL upregulation, were completely prevented by the PTK inhibitor genistein. The activation of protein kinase C (PKCδ, ε, and μ) was also induced following aburatubolactam C treatment. Although the activation of p56^{lck} and tyrosine phosphorylation of the cellular proteins were not blocked by the PKC inhibitor GF109203X, the activation of ERK2 was completely abrogated, along with a detectably enhanced JNK1 activation, FasL upregulation, and apoptosis. However, the FasL upregulation and apoptosis were significantly inhibited by the PKC activator PMA, with a remarkable increase in the ERK2 activation. The cytotoxic effect of aburatubolactam C was reduced in the presence of the anti-Fas neutralizing antibody ZB-4. Although ectopic expression of Bcl-2 failed to completely block the cytotoxicity of aburatubolactam C, it was clearly suppressed. The c-Fos mRNA expression was upregulated in a biphasic manner, where the second phasic expression overlapped with the FasL upregulation. Accordingly, these results demonstrate that aburatubolactam C-induced apoptosis is exerted, at least in part, by FasL upregulation dictated by activation of the PTK (p56^{lck} and p59^{fyn})/JNK1 pathway, which is negatively affected by the concurrent activation of the PKC/ERK2 pathway proximal to PTK activation.

Key words: Aburatubolactam C, PTK activator, apoptosis, FasL-upregulation, PKC/ERK signaling pathway, PTK/JNK signaling pathway

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Although apoptosis can be induced as a response to numerous physiological and nonphysiological signals such as T cell receptor (TCR) engagement, the tumor necrosis factor (TNF), Fas ligation, oxidative stress, growth factor withdrawal, corticosteroids, heat shock, irradiation, and chemotherapeutic agents, Fas-mediated apoptosis upon interaction with Fas ligand (FasL) is critically involved in the elimination of cells *in vivo* [1, 13, 15, 21, 34]. Several tumor cells have been known to escape Fas-mediated cell death by altering the expression of either Fas [6, 30] or its downstream signaling mediators [22]. This disruption of the apoptosis pathway and consequent imbalance between the apoptotic and mitotic rate can then confer a survival advantage causing tumor progression. Since chemotherapy for tumors is principally based on agents that are toxic to the cells and since induction of apoptosis in tumor cells can lead to their own destruction, apoptosis has been currently suggested as an efficient mechanism by which malignant tumor cells are removed when treated with antineoplastic drugs. This is because a potential mechanism implicated in

drug-induced apoptosis is the upregulation of FasL and Fas expressions with the subsequent induction of apoptotic cell death through the activation of Fas signaling [12, 14, 24, 25].

The majority of studies on Fas-mediated apoptosis have focused on T cells and revealed that Fas plays an important regulatory role in T cell homeostasis as well as periphery tolerance and T cell-mediated cytotoxic reactions [13, 21]. It has been reported that a src-family protein tyrosine kinase (PTK) $p56^{lck}$ and ZAP-70 tyrosine kinase are required for TCR-mediated FasL expression and thus the development of activation-induced T cell apoptosis [10, 26]. It has also been shown that a src-related PTK $p59^{fyn}$ is physically associated with Fas and may contribute to Fas signal transduction in activated T cells [2]. Recently, there have been reports regarding the involvement of the coordinated action of protein kinase C (PKC) and calcineurin, and the MAP kinase cascade in the pathway that induces FasL expression in T cells, following TCR/CD3 stimulation [38, 41]. These results indicate that the $p56^{lck}$, $p59^{fyn}$, and ZAP-70 tyrosine kinases as well as their downstream signaling mediators PKC, calcineurin, and MAP kinases may be critically involved in the apoptotic death accomplished by the Fas/FasL system in T cells. Nonetheless, the molecular mechanisms underlying the upregulation of FasL or Fas expression in tumors by chemotherapeutic agents are still poorly understood.

Aburatubolactam C, a new lactam compound with the molecular formula of $C_{30}H_{40}N_2O_5$ and molecular mass of 508, was originally isolated from a marine *Streptomyces* sp. as a novel inhibitor of superoxide anionic generation in human neutrophils [4]. In a previous study, we showed that aburatubolactam C is cytotoxic for various tumor cells of human and murine origin [5]. A subsequent investigation of the mechanism responsible for the cytotoxicity revealed that aburatubolactam C induces apoptotic death in Jurkat T cells possibly through the Fas/FasL system [3]. Accordingly, the present study further investigated the molecular signaling pathway underlying the upregulation of FasL expression in Jurkat T cells following aburatubolactam C-treatment, along with determining the functional role of aburatubolactam C as a PTK activator. In addition, the effects of the anti-Fas neutralizing antibody ZB-4 and anti-apoptotic protein Bcl-2 on the cytotoxicity of aburatubolactam C were investigated to confirm the involvement of the FasL/Fas system in the cytotoxicity and examine the potency of aburatubolactam C as an antineoplastic agent.

MATERIALS AND METHODS

Reagents, Antibodies, and Cells

The aburatubolactam C was kindly provided by Dr. Kaoru Yamada (Sagami Chemical Research Center, Kanagawa, Japan). The genistein, a specific PTK inhibitor, was purchased from Life Technologies (Gaithersburg, MD, U.S.A.) and the GF109203X was obtained from BIOMOL Research Labortories (Plymouth, PA, U.S.A.). The radioactive materials including [^{3}H]thymidine (2 Ci/mmol), and ^{125}I-rabbit anti-mouse IgG were from NEN Biotechnology System (Boston, MA, U.S.A.), and [γ-^{32}P]ATP (~3,000 Ci/mmol) was from Amersham (Arlington Heights, IL, U.S.A.). The anti-phosphotyrosine antibody PY-20 was purchased from ICN Biomedicals (Costa Masa, CA, U.S.A.). The monoclonal anti-FasL, anti-Fas, anti-$p56^{lck}$, anti-$p59^{fyn}$, and anti-ERK2 antibodies were purchased from Transduction Laboratories (Lexington, KY, U.S.A.). The isoform-specific anti-PKC monoclonal antibodies for β, δ, ε, θ, and μ were obtained from Transduction Laboratories (Lexington, KY, U.S.A.) and the polyclonal antibody for α was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The monoclonal anti-phosphorylated ERK2 was purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). The monoclonal anti-phosphorylated JNK1 and anti-phosphorylated p38 antibodies, and polyclonal anti-JNK1, anti-p38, anti-$p56^{lck}$, and anti-$p59^{fyn}$ antibodies were obtained from Santa Cruz Biotechnology. The anti-Fas neutralizing antibody ZB-4 and Fas-agonistic antibody CH-11 were both obtained from Upstate Biotechnology. The human acute leukemia Jurkat T cells were kindly supplied by Dr. Albert A. Nordin (Gerontology Research Center, NIA/NIH, Baltimore, MD, U.S.A.). The stable transfectant of Jurkat T cells with Bcl-2 was provided by Dr. Dennis Taub (Gerontology Research Center, NIA/NIH, Baltimore, MD, U.S.A.). The Jurkat T cells were cultured in RPMI 1640 containing 10% FBS, 20 mM HEPES (pH 7.0), 5×10^{-5} M 2-ME, and 100 μg/ml gentamycin. The Bcl-2- and Bcl-xL-stable transfectants were maintained in RPMI1640 medium supplemented with 500 μg/ml of G418.

Cytotoxicity Assay

The cytotoxic effect of aburatubolactam C on Jurkat T cells was analyzed by MTT assay, reflecting the cell viability. For the MTT assay, Jurkat T cells (5×10^4) or human peripheral T cells (1.5×10^5) were added to a serial dilution of aburatubolactam C in 96-well plates. After incubation for 24 h, 50 μl of the MTT solution (1.1 mg/ml) was added to each well and the plate was incubated for an additional 4 h. After centrifugation, the supernatant was removed from each well, and then 150 μl of DMSO was added to dissolve the colored formazan crystal produced from MTT. The absorbance of the solution was measured at 540 nm using a plate reader. To examine the suppressive effect of the anti-Fas neutralizing antibody ZB-4 on the cytotoxicity of the Fas agonistic antibody or aburatubolactam C, Jurkat T cells (5×10^4) were pretreated for 1 h using 250 or 500 ng/ml of anti-Fas neutralizing antibody ZB-4 in 96-well plates. The cells were then treated with either 50 ng/

ml of the cytotoxic anti-Fas antibody CH-11 or 3 μg/ml of aburatubolactam C for 48 h. The cell viability was analyzed by MTT assay.

DNA Fragmentation Analysis

The apoptotic DNA fragmentation, induced in the Jurkat T cells following the treatment with the external stimulants, was determined as previously described [18]. Briefly, the cells were harvested by centrifugation and then treated with a lysis buffer (0.5% Triton X-100, 5 mM EDTA, 10 mM Tris-HCl, pH 7.4) for 20 min on ice. After centrifugation for 15 min at 14,000 rpm, the supernatant was collected and treated for 2 h at 50°C with proteinase K and subsequently with RNase for 4 h at 37°C. After extraction with an equal volume of buffer-saturated phenol, the DNA fragments were precipitated with 2.5 volumes of ethanol in the presence of 0.5 M NaCl and visualized following electrophoresis on a 1.2% agarose gel.

Cell Fractionation

The separation of the cytosolic and particulate membrane fractions was performed as described previously [7]. Briefly, Jurkat T cells treated with aburatubolactam C for the indicated time periods were suspended in buffer A (20 mM Tri-HCl, 0.25 M sucrose, 2 mM EGTA, 2 mM EDTA, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM PMSF, pH 7.5). The cells were then sonicated for 6s twice, centrifuged at 100,000 ×*g* for 1 h, and the supernatant was designated as the cytosolic fraction. Thereafter, the pellet was extracted using buffer B (20 mM Tris-HCl, 1% SDS, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM PMSF, pH 7.5), centrifuged 15,000 ×*g* for 15 min, and the supernatant was saved as the particulate membrane fraction.

Western Blot Analysis

Cell lysates were prepared by suspending 5×10^6 Jurkat T cells in 100 μl of lysis buffer (20 mM Tris-HCl, 137 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM sodium orthovanadate, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM PMSF, 1% NP-40, pH 8.0). The cells were then disrupted by sonication and extracted at 4°C for 30 min. Next, equivalent amounts of the cell lysates were subjected to electrophoresis on 8–16% gradient SDS polyacrylamide gels unless described in the conditions, and electrotransferred to Immobilon-P membranes (Millipore Co., Bedford, MA, U.S.A.). The membranes were allowed to react with the individual primary antibodies and then with horseradish peroxidase conjugated sheep anti-mouse IgG or donkey anti-rabbit IgG antibody. The detection of each protein was visualized on X-ray film using an ECL Western blotting detection system according to the manufacturer's instructions.

Immunoprecipitation and Kinase Assay

For the immunoprecipitation of $p56^{lck}$ and $p59^{fyn}$, 70 μg of the cellular lysate at 1 μg/ml were allowed to react with 2 μg of each antibody at 4°C for 2 h. The immune complexes were recovered by the addition of 30 μl of protein G-agarose, and the incubation was continued with rotation at 4°C for 1 h. The protein G-agarose was collected by centrifugation (2,000 ×*g*) for 3 min, then washed three times with the lysis buffer and once with the kinase assay buffer. The α-casein kinase activity of the immunoprecipitated $p56^{lck}$ and $p59^{fyn}$ was assayed as previously described [20]. The phosphorylation of α-casein was measured by incubating the protein G-agarose beads with 30 μl of a kinase assay cocktail (15 μg of α-casein, 10 μCi [γ-^{32}P]ATP, 100 mM ATP, 1× kinase assay buffer) for 30 min at 30°C. The reaction was stopped by boiling the mixture in 1× SDS sample buffer for 5 min, and the reaction mixture was resolved on an 11% SDS-polyacrylamide gel electrophoresis. Thereafter, the gel was dried and phosphorylation of the casein was detected by autoradiography and quantitated using a phosphoimage analyzer.

Northern Blot Analysis

The total RNA was extracted and isolated by solubilization in guanidine thiocyanate as described elsewhere [20]. Equivalent amounts of the total RNA were electrophoresed on 1% formaldehyde-agarose gels and transferred to GeneScreen Plus membranes. The nylon membranes were then hybridized in an Express Hyb solution at 68°C for 2 h with a cDNA probe radiolabeled with [^{32}P] dCTP using the random primer labeling method, and washed according to the manufacturer's instruction.

RESULTS

Direct Activation of Protein Tyrosine Kinases $p56^{lck}$ and $p59^{fyn}$ by Aburatubolactam C *In Vitro*

The aburatubolactam C-induced apoptotic DNA fragmentation in the human Jurkat T cells appeared to occur concurrently with FasL upregulation and an enhanced tyrosine phosphorylation of multiple cellular proteins. These apoptotic cellular changes were blocked by the PTK inhibitor genistein, indicating the requirement of PTK activation for the drug-induced *FasL upregulation and apoptosis* [3]. Since the PTK associated with the aburatubolactam C-induced FasL upregulation accompanying apoptosis was expected to be the src-related PTKs $p56^{lck}$ and $p59^{fyn}$, which are abundantly expressed in T cells, the kinetic alteration of the kinase activity of $p56^{lck}$ and $p59^{fyn}$ in the Jurkat T cells following the aburatubolactam C treatment was assessed by an *in vitro* kinase assay using α-casein as the substrate (Figs. 1A and 1B). Although the time points for the activation of the

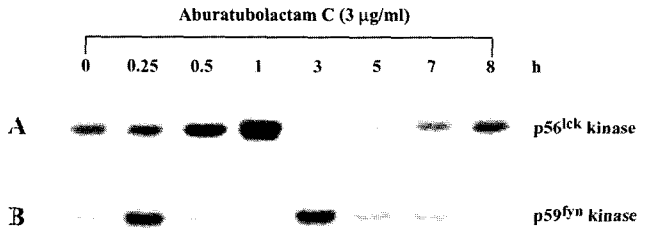

Fig. 1. Activation time course for $p56^{lck}$ (A) and $p59^{fyn}$ (B) in Jurkat T cells following addition of aburatubolactam C.
Continuously growing Jurkat T cells ($4-6\times10^6$) were incubated at a concentration of 5×10^5/ml with 3 μg/ml of aburatubolactam C for the indicated times and the cells were collected to prepare cell lysates. Equivalent amounts of the cell lysate (70 μg) were then immunoprecipitated with each antibody and the immune complex was subjected to a kinase assay. The kinase reaction mixture was electrophoresed on an 11% SDS-polyacrylamide gel, which was then dried, and the phosphorylated substrate was visualized by autoradiography after exposure at −70°C.

individual kinases appeared to be different, their activation patterns were biphasic. The casein kinase activity of $p56^{lck}$, which significantly increased during the first hour, declined to a barely detectable level after 3 h, and then gradually increased to a basal level after 8 h. Meanwhile, $p59^{fyn}$ was transiently activated at two different time points of 15 min and 3 h after the addition of aburatubolactam C. Therefore, these results indicate that the PTKs $p56^{lck}$ and $p59^{fyn}$ were activated in the Jurkat T cells by aburatubolactam C.

To understand the mechanism accounting for the rapid activation of $p56^{lck}$ and $p59^{fyn}$ in the aburatubolactam C-treated Jurkat T cells, the direct activation of the tyrosine kinases by aburatubolactam C was investigated *in vitro*. The $p56^{lck}$ and $p59^{fyn}$ in the Jurkat T cells were recovered by immunoprecipitation and their casein kinase activities were assayed in the presence of various concentrations of aburatubolactam C. The $p56^{lck}$ was found to be activated by aburatubolactam C within a range of 1–4 μg/ml and exhibited an approximately 2.4-fold increase in activity with a maximum at 1 μg/ml (Fig. 2A), whereas $p59^{fyn}$ was activated within a range of 0.5–4 μg/ml with a maximum 2.2-fold increase at 2 μg/ml of aburatubolactam C (Fig. 2B), demonstrating that the activation of $p56^{lck}$ and $p59^{fyn}$ induced in the aburatubolactam C-treated Jurkat T cells was due to direct activation of the kinases by the aburatubolactam C in the cells.

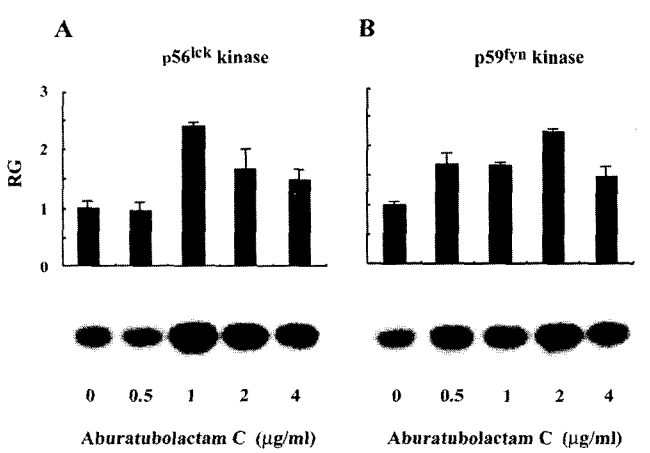

Fig. 2. Effect of aburatubolactam C on casein kinase activity of $p56^{lck}$ (A) and $p59^{fyn}$ (B) immunoprecipitated from Jurkat T cells.
Continuously growing Jurkat T cells were harvested and cell lysates were prepared. Equivalent amounts of the cell lysates (70 μg) were then immunoprecipitated with anti-$p56^{lck}$ or anti-$p59^{fyn}$ antibodies and the immune complex was assayed for α-casein kinase activity in the presence of various concentrations of aburatubolactam C. The kinase reaction mixture was electrophoresed on an 11% SDS-polyacrylamide gel. The gel was then dried and the phosphorylated α-casein was visualized by autoradiography after exposure at −70°C. Quantification of the autoradiograms was performed using a phosphoimage analyzer (Kodak).

Kinetics of Activation of Protein Kinases Following Aburatubolactam C Treatment

To further understand the aburatubolactam C-induced signaling pathway leading to FasL upregulation, the kinetic changes in the activity level of MAP kinase family members including ERK, JNK1, and p38 have also been assessed based on recent studies, which suggested that a signaling balance between the TCR-mediated activation of ERK and JNK activation by Fas may be important in determining whether T cells survive or undergo apoptosis [40]. In

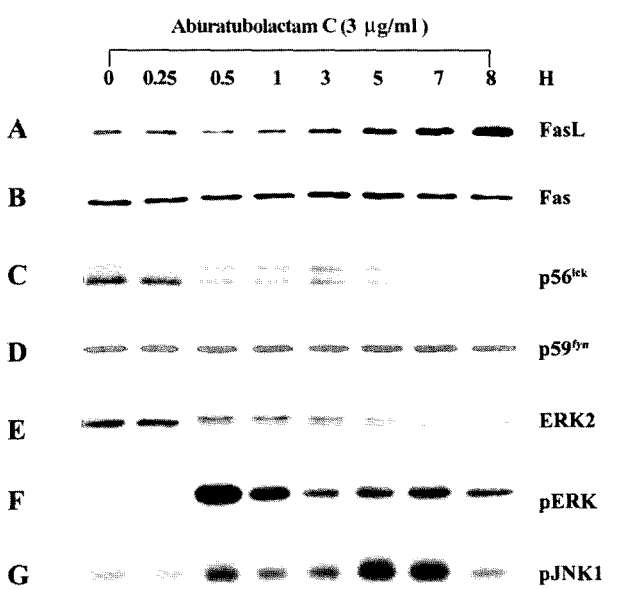

Fig. 3. Kinetic analysis of aburatubolactam C-induced change in protein level of FasL (A) and Fas (B), electrophoretic mobility shift of $p56^{lck}$ (C), $p59^{fyn}$ (D), and ERK2 (E), and expression of the active phosphorylated form of ERK2 (F) and JNK1 (G) in Jurkat T cells.
Equivalent amounts of the cell lysate (70 μg) prepared as described in Fig. 1 were electrophoresed on 8–16% SDS gradient polyacrylamide gels and electrotransferred to Immobilon-P. Western blot analysis was performed as described in Materials and Methods.

the current study, when Jurkat T cells were treated with aburatubolactam C at a concentration of 3 μg/ml, an increased FasL protein level was first detected after 3 h, which then continued to increase until 8 h, yet no change was detected in the Fas protein level (Figs. 3A and 3B). Under these conditions, the maximum level of electrophoretic mobility shift from $p42^{ERK2}$ to $p44^{ERK2}$ was induced after 30 min and sustained until 7 h (Fig. 3E). The slow moving $p44^{ERK2}$ was also determined to be the active phosphorylated form of $p42^{ERK2}$, which was recognizable using anti-pERK2 (Fig. 3F). In addition to the activation of ERK2, the active phosphorylated form of JNK1 began to increase after 30 min and reached a maximum 5–7 h after the addition of aburatubolactam C (Fig. 3G). A mobility shift from $p56^{lck}$ to $p60^{lck}$, which is known to reflect a rapid and significant activation of $p56^{lck}$ [37], was also detected in accordance with the data in Fig. 1, yet no changes were detected in either the protein level or electrophoretic mobility of $p59^{fyn}$ (Figs. 3C and 3D). No active phosphorylated form of MAP kinase p38 was detected (data not shown). Therefore, these results indicate that the PTKs $p56^{lck}$ and $p59^{fyn}$ and MAP kinases ERK2 and JNK1 were activated in the Jurkat T cells by aburatubolactam C prior to the upregulation of FasL expression, suggesting that these kinases may also be involved in the drug-induced signaling pathway leading to FasL upregulation and apoptotic DNA fragmentation.

To further elucidate the involvement of the PTKs $p56^{lck}$ and $p59^{fyn}$ and MAP kinases ERK2 and JNK1 and their relevant sequence in relaying the signal required for aburatubolactam C-induced FasL upregulation, the effect of the specific PTK inhibitor genistein on the aburatubolactam C-induced activation of these protein kinases in the Jurkat T cells was investigated. As shown in Figs. 4A and 4B, the presence of 200 μM genistein appeared to block the aburatubolactam C-induced FasL upregulation, although there was no detectable change in the protein level of Fas. In addition, there was no mobility shift of $p56^{lck}$ to $p60^{lck}$ in the gel or activation of the MAP kinases ERK2 and JNK1 (Figs. 4C–4E). These results indicate that the drug-induced activation of $p56^{lck}$ and $p59^{fyn}$, which is a prerequisite for FasL upregulation, precedes the activation of both ERK2 and JNK1 in the Jurkat T cells following aburatubolactam C-treatment.

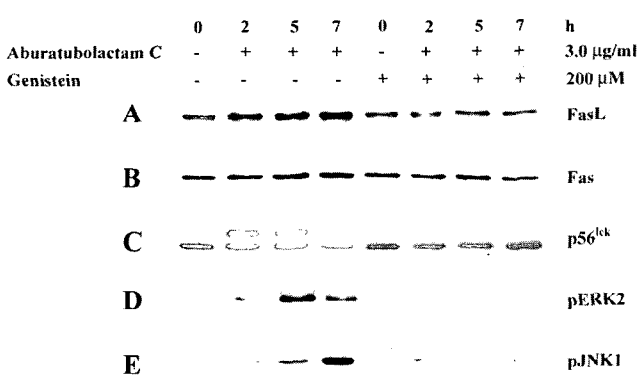

Fig. 4. Effect of genistein on aburatubolactam C-induced change in protein level of FasL (A) and Fas (B), and electrophoretic mobility retardation of $p56^{lck}$ (C), phosphorylated ERK2 (D), and phosphorylated JNK1 (E) in Jurkat T cells.
After pretreatment with 200 μM of genistein for 1 h, the treated and untreated cells were incubated with 3 μg/ml of genistein for the indicated times, and the cells were harvested to prepare cell lysates. Equivalent amounts of the cell lysate (70 μg) were electrophoresed on an 8–16% SDS gradient polyacrylamide gel and electrotransferred to Immobilon-P. Western blot analysis was performed as described in Materials and Methods.

Role of PKC in Aburatubolactam C-Induced Signaling Pathway and Apoptosis

Since the activation of the PTKs seemingly occurred prior to the activation of ERK2 and JNK1 during the aburatubolactam C-induced FasL upregulation in the Jurkat T cells, the potential role of PKC in the drug-induced signaling pathway leading to FasL upregulation was examined by monitoring the activation of individual PKC isozymes based on measuring their translocations from the cytosol to the particulate membrane fraction in the aburatubolactam C-treated Jurkat T cells. As determined by Western analysis with specific antibodies, the Jurkat T cells were found to express multiple PKC isoforms such as conventional PKC (cPKCα, βI, and βII), novel PKC (nPKCδ, ε, and θ), and atypical PKC (aPKC, μ) (Fig. 5). Although there was a slight decrease in the level of all

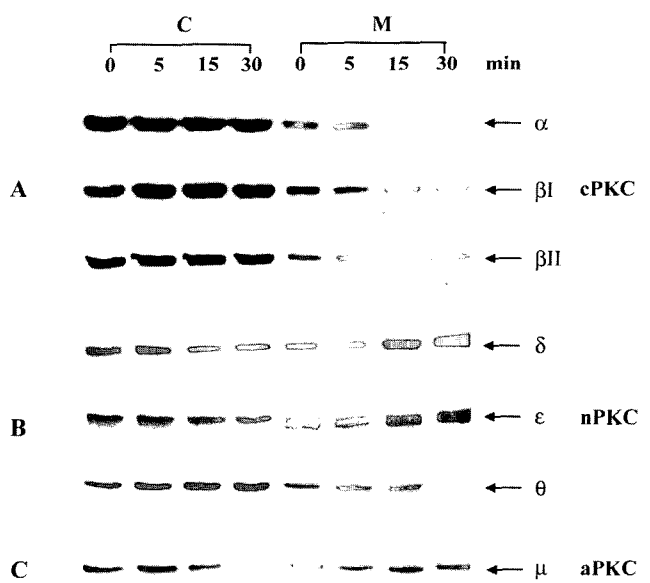

Fig. 5. Effect of aburatubolactam C on the distribution of PKC isozymes in Jurkat T cells.
The cells were treated with 3 μg/ml of aburatubolactam C for the indicated times and fractionated into the cytosolic (C) and particulate membrane (M) fractions. Equivalent amounts of the individual fractions were then electrophoresed on 10% polyacrylamide gels and electrotransferred to Immobilon-P. Western blot analysis was performed as described in Materials and Methods.

cPKCs tested and nPKCθ in the particulate membrane fraction of the Jurkat T cells within 30 min following aburatubolactam C-treatment, a translocation of nPKC (δ and ε) and aPKCμ was detected, demonstrating that nPKCδ and ε, and aPKCμ could be rapidly activated and may play a role in the aburatubolactam C-induced signaling pathway and apoptotic DNA fragmentation.

To further examine the role of PKC in the apoptotic cellular changes, the effect of GF109203X, a known PKC inhibitor acting on the catalytic site [19], on the aburatubolactam C-induced activation of signaling, FasL upregulation, and apoptotic DNA fragmentation was investigated. As shown in Fig. 6A, the apoptotic DNA fragmentation of the Jurkat T cells induced by 1.5 μg/ml of aburatubolactam C appeared to be enhanced in the presence of 5 and 10 μM of GF109203X in a dose-dependent manner. Although there was a slight decrease in the level of aburatubolactam C-induced tyrosine phosphorylation of the multiple cellular proteins ranging in molecular mass from 32 to 120 kDa and typical electrophoretic mobility shift from $p56^{lck}$ to $p60^{lck}$, these cellular changes induced by aburatubolactam C (1.5 μg/ml) were still clearly detectable in the presence of 10 μM of GF109203X (Figs. 6B and 6D). In accordance with an increased apoptotic DNA fragmentation, the aburatubolactam C-induced FasL upregulation and JNK1 activation appeared to be enhanced by GF109203X (Figs. 6C and 6F). However, under the same conditions, ERK2 activation in response to aburatubolactam C was completely abrogated, demonstrating that the activation of ERK2 was a downstream target of the drug-induced PTK/PKC activation pathway, but not an essential signal for the upregulation of FasL expression and induction of apoptosis (Fig. 6E). Furthermore, these results indicate that the activation of JNK1, which also acts as a downstream signal of the drug-induced PTK activation required for FasL upregulation, did not occur through the activation of PKC, and that the aburatubolactam C-induced PTK/PKC/ERK2 activation pathway may negatively modulate the induced PTK/JNK1 activation pathway leading to the upregulation of FasL expression and apoptotic DNA fragmentation.

Since the aburatubolactam C-induced apoptotic DNA fragmentation and FasL upregulation were apparently promoted

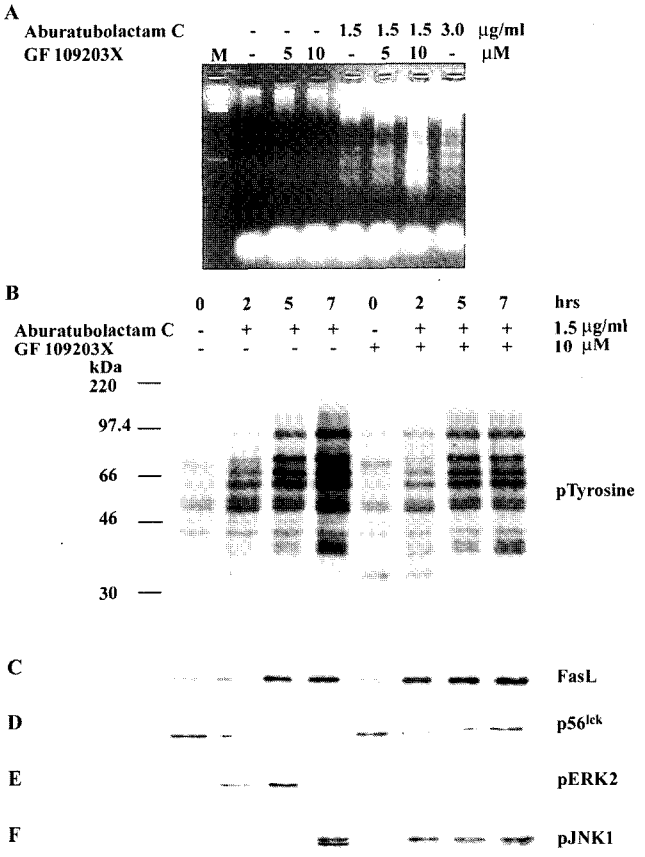

Fig. 6. Effect of GF109203X on aburatubolactam C-induced apoptotic DNA fragmentation (A), tyrosine phosphorylation of cellular proteins (B), upregulation in the protein level of FasL (C), and electrophoretic mobility retardation of $p56^{lck}$ (D), phosphorylated ERK2 (E), and JNK1 (F) in Jurkat T cells.
For the apoptotic DNA fragmentation analysis, cells pretreated with 5 or 10 μM of GF109203X for 1 h were incubated with 1.5 μg/ml of aburatubolactam C for 7 h. For Western blot analysis, cells pretreated with 10 μM of GF109203X for 1 h were incubated with 1.5 μg/ml of aburatubolactam C for the indicated times. The cells were processed to assess the tyrosine phosphorylation of the cellular proteins, change in the FasL expression, electrophoretic mobility shift of $p56^{lck}$, and active phosphorylated form of ERK2 and JNK1 by Western blot analysis.

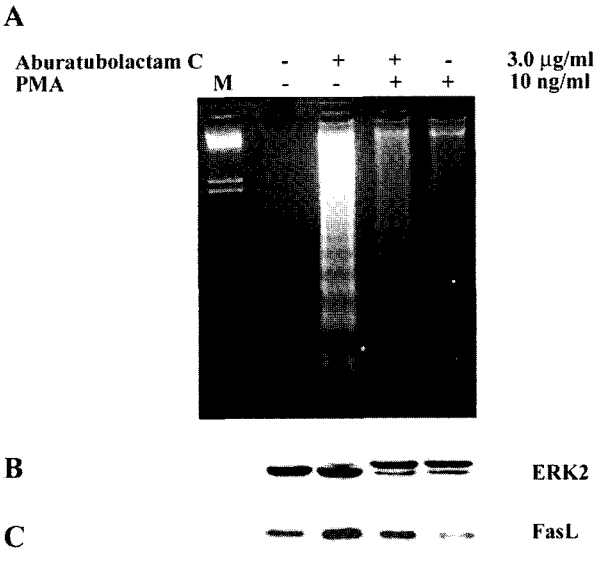

Fig. 7. Effect of PMA on aburatubolactam C-induced apoptotic DNA fragmentation (A), electrophoretic mobility shift of ERK2 (B), and upregulation of FasL protein level (C) in Jurkat T cells.
After the cells were incubated with 3 μg/ml of aburatubolactam C in the presence of 10 ng/ml of PMA for 7 h, the cells were processed to either analyze the apoptotic DNA fragmentation or assess the electrophoretic mobility shift of ERK2 and FasL protein level using Western blot analysis.

in the presence of the PKC inhibitor GF109203X, the effect of PKC activation on the drug-induced apoptotic DNA fragmentation and FasL upregulation was then examined using a known PKC activator, PMA. As shown in Fig. 7, the FasL upregulation accompanying apoptotic DNA fragmentation in the Jurkat T cells induced by 3.0 μg/ml of aburatubolactam C was significantly inhibited by the addition of 10 ng/ml of PMA. The PMA treatment also significantly promoted the mobility shift of $p42^{ERK2}$ to $p44^{ERK2}$, reflecting an enhanced activation of ERK2. Consequently, these results confirmed that the aburatubolactam C-induced PKC/ERK2 activation pathway, proximal to PTK activation, plays a negative role in the drug-induced upregulation of FasL.

Suppression of Cytotoxicity of Aburatubolactam C by Anti-Fas Neutralizing Antibody ZB-4

Based on the consistent and concomitant occurrence of aburatubolactam C-induced apoptotic DNA fragmentation and upregulation of FasL, the critical role of the upregulation of FasL and subsequent induction of Fas-mediated apoptotic cell death in the cytotoxicity of aburatubolactam C in Jurkat T cells was confirmed by examining whether the anti-Fas neutralizing antibody ZB-4 could block the cytotoxicity of aburatubolactam C. ZB-4 has been previously employed to block the cytotoxic effect of Fas agonistic antibodies [16, 23]. Pretreatment using ZB-4 (500 ng/ml) followed by the cytotoxic anti-Fas antibody CH-11 (50 ng/ml) resulted in an almost complete blockage of CH-11-induced cytoxicity in the Jurkat T cells (Fig. 8A). Under these conditions, although the cytotoxic effect of aburatubolactam C (3 μg/ml) was not completely blocked, ~50% of the cytotoxicity was apparently reduced by ZB-4, confirming that the Fas/FasL system was associated with the aburatubolactam C-induced apoptotic cell death of the Jurkat T cells (Fig. 8B).

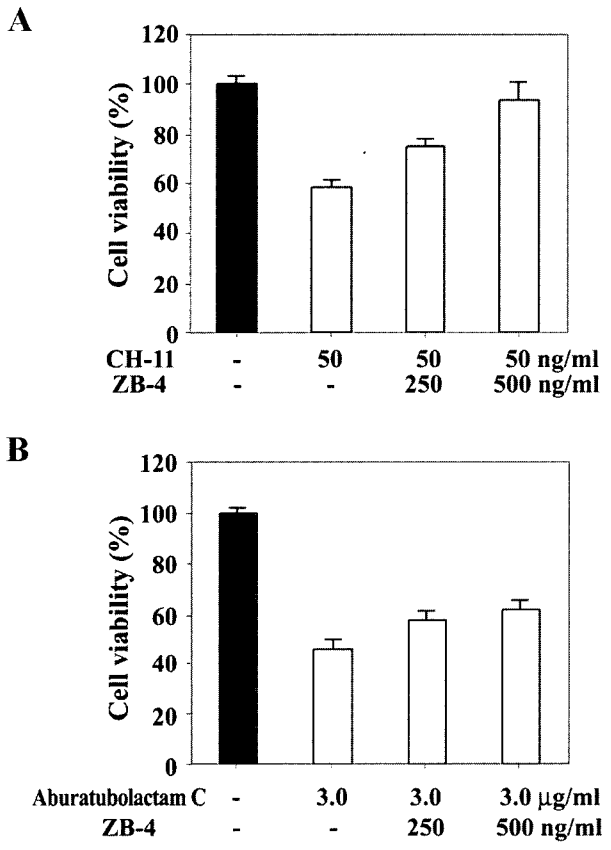

Fig. 8. Effect of anti-Fas neutralizing antibody ZB-4 on anti-Fas agonistic antibody CH-11 (A) or aburatubolactam C-mediated cytotoxicity (B) in Jurkat T cells.

In 96-well plates, Jurkat T cells were pretreated for 1 h using 250 and 500 ng/ml of ZB-4, and then challenged with either the anti-Fas agonistic antibody CH-11 (50 ng/ml) or aburatubolactam C (3 μg/ml). After 20 h, an MTT assay was performed to determine the cell viability.

Effect of Ectopic Overexpression of Bcl-2 on Cytotoxicity of Aburatubolactam C

It has been reported that the dysregulation of normal apoptotic mechanisms by the overexpression of anti-apoptotic Bcl-2 family members, such as Bcl-2 and its functional homolog Bcl-xL, often provides a growth advantage and apoptotic resistance against various apoptosis-inducing stimuli to cells. Although the anti-apoptotic proteins Bcl-2 and BcL-xL can protect cells from apoptosis induced by diverse signals, such as ionizing radiation, hypoxia, or chemotherapeutic agents [8, 31, 32, 36], the protection of Bcl-2 or Bcl-xL against Fas-transduced apoptosis appears to be controversial, depending on the cell types. A study of various cell lines has shown that Bcl-2 can inhibit Fas-induced apoptosis in certain cells (Type II), yet not others (Type I) [29]. Therefore, since Jurkat T cells are Type II cells and the current results indicated that aburatubolactam C induced apoptosis in Jurkat T cells through FasL upregulation and subsequent mediation of Fas death signaling, the ability of Bcl-2 to interfere with aburatubolactam C-induced apoptosis was investigated by examining the effect of the ectopic expression of Bcl-2 on the drug-induced cytotoxicity in Jurkat T cells. As shown in Fig. 9A, the transfectant of the Bcl-2 gene was able to express a significantly increased level of Bcl-2 protein in the Jurkat T cells. Under these conditions, the overexpressed Bcl-2 completely blocked the cytotoxic effect of Fas against the antibody CH-11 in the Jurkat T cells, indicating that the overexpressed Bcl-2 was functionally active (Fig. 9B). Although the protection from the cytotoxicity of aburatubolactam C was not as pronounced in the Jurkat T cells as that observed with the CH-11-induced cytotoxicity, a clear blocking effect was still detected (Fig. 9C), supporting the premise that the aburatubolactam C-induced apoptosis in the Jurkat T cells was conducted, at least in part, by FasL upregulation and the subsequent mediation of Fas death signaling, which was interrupted by Bcl-2.

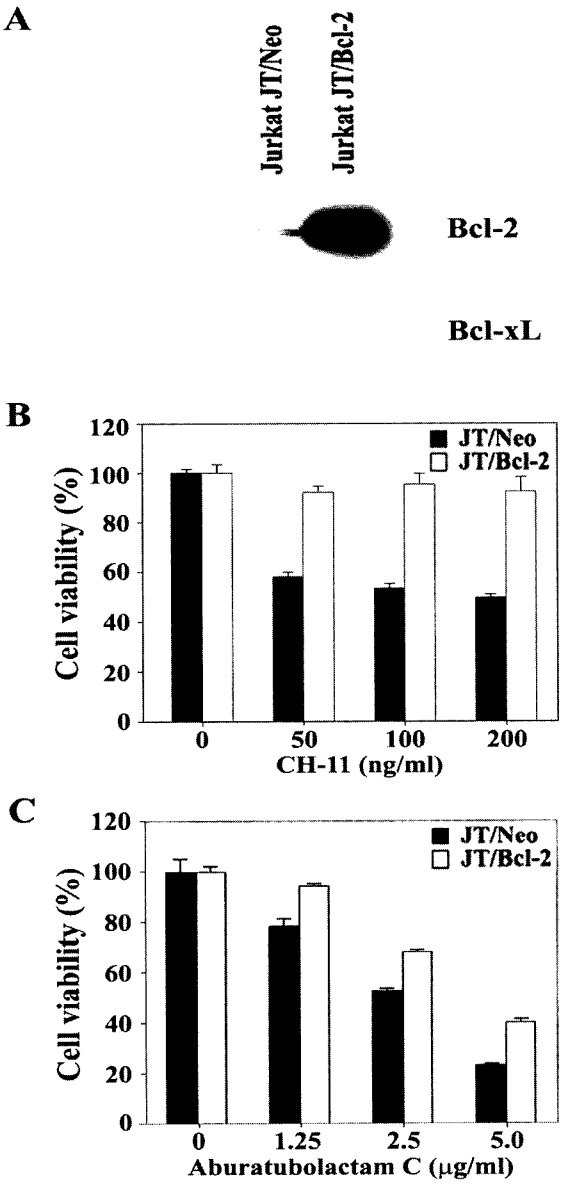

Fig. 9. Effect of Bcl-2 on cytotoxicity of aburatubolactam C in Jurkat T cells.
The ectopic overexpression of the Bcl-2 protein in Jurkat T cells transfected with the Bcl-2 gene construct was confirmed by Western blot analysis (A). Jurkat T cells overexpressing Bcl-2 (JT/Bcl-2) or control cells (JT/Neo) were incubated at a density of 5×10^4/well with various concentrations of CH-11 (B) or aburatubolactam C (C) in 96-well plates. After incubation for 20 h, MTT was added for an additional 4 h before the cells were harvested to assess the colored formazan crystal produced from the MTT as an index of cell viability.

Upregulation of mRNA Level Specific for c-Fos Following Treatment with Aburatubolactam C

In a previous study, it was reported that the apoptotic upregulatoin of FasL in hepatoma cells following antineoplastic drugs was dictated by the activation of a JNK signaling pathway, as well as a novel AP-1 element in the FasL promoter, and the identified AP-1 site in the FasL promoter

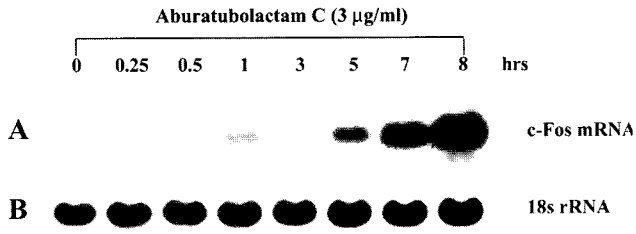

Fig. 10. Northern blot analysis of c-Fos-specific mRNA i Jurkat T cells following treatment with aburatubolactam C.
Continuously growing Jurkat T cells ($4-6\times10^6$) were incubated at concentration of 5×10^5 ml with 3 μg/ml of aburatubolactam C for th indicated times and the cells were collected to extract the total RNA. Te micrograms of total RNA from each time point was electrophoresec transferred, and probed with ^{32}P-labeled c-Fos (A), and 18S rRNA (B) cDNA

was recognized by the Jun-Fos heterodimer [9]. Accordingly since the current data indicated that the aburatubolactan C-induced FasL upregulation was dictated by the activatio of the PTKs ($p56^{lck}$ and $p59^{fyn}$)/JNK1 pathway, it was decide to investigate the change in the expression level of c-Fo mRNA in Jurkat T cells after addition of aburatubolactar C. As shown in Fig. 10, when Jurkat T cells were treate with 3 μg/ml of aburatubolactam C, the level of c-Fo mRNA, which began to increase after 30 min, declined t a barely detectable level after 3 h and then significantl increased until 8 h, indicating that the upregulation patter for c-Fos mRNA expression was biphasic. In particular, th time points for the aburatubolactam C-induced upregulatio of c-Fos mRNA appeared to be essentially the same a those for JNK1 activation, and the second phasic upregulatio of c-Fos mRNA expression was consistent with the Fas upregulation, suggesting the possible involvement of bot the activation of AP-1 via the upregulation of c-Fos mRN and the JNK1 activation pathway in the aburatubolactar C-mediated FasL upregulation.

DISCUSSION

The autocrine and/or paracrine activation of Fas signalin caused by the upregulation of FasL and Fas expression is potential mechanism for chemotherapeutic drug-induce apoptosis. The molecular mechanisms underlying the induce upregulation of Fas and FasL expression and apoptos upon exposure to anticancer drugs are of major importanc in understanding the efficacy of cytotoxic drugs in killin tumor cells. Previously, we hypothesized that the apoptos of human Jurkat T cells induced by the cytotoxic dru aburatubolactam C involves the interaction of Fas wit FasL [3]. The present study confirmed the association c the Fas/FasL system with the aburatubolactam C-induce apoptotic death of Jurkat T cells, along with the molecul signaling pathway leading to the upregulation of FasL. I

this regard, the changes in the activities of protein kinases, such as the src-associated PTKs $p56^{lck}$ and $p59^{fyn}$, and MAP kinase members ERK2, JNK1, and p38, were investigated. The PTKs $p56^{lck}$ and $p59^{fyn}$ and MAP kinases ERK2 and JNK1 were all found to be activated well before the upregulation of FasL expression. Among these kinases, the activation of $p59^{fyn}$ occurred within 15 min as the earliest event. In particular, the aburatubolactam C-induced activation of $p59^{fyn}$ and JNK1 was biphasic in time and their second phasic activations overlapped with the FasL upregulation. However, there was no dectectable activation of the MAP kinase p38 (data not shown), indicating that the p38 activation pathway was not associated with the aburatubolactam C-induced FasL upregulation or onset of cell death. Consequently, these results suggest that the rapid activation of $p56^{lck}$, $p59^{fyn}$, ERK2, and JNK1 in the early stage contributed to the signaling pathway leading to FasL upregulation, whereas the second phasic activation of $p59^{fyn}$ and JNK1 was more likely involved in the Fas death signaling. Since the activation of PTK $p56^{lck}$, which is an immediate early event for T cell activation through TCR, contributes to FasL expression for activation-induced T cell death [10, 26], the present study also investigated whether aburatubolactam C would act as an activator for the PTKs $p56^{lck}$ and $p59^{fyn}$ *in vitro*. The casein kinase activity of the immunoprecipitated $p56^{lck}$ or $p59^{fyn}$ from Jurkat T cells was found to be augmented by the presence of aburatubolactam C in the range of 1–4 μg/ml for $p56^{lck}$ and 0.5–4 μg/ml for $p59^{fyn}$, demonstrating that direct activation by aburatubolactam C was the mechanism responsible for the drug-induced activation of $p56^{lck}$ and $p59^{fyn}$. Conversely, the drug-induced cellular changes, including activation of $p56^{lck}$, ERK2, and JNK1, and FasL upregulation were prevented by the specific PTK inhibitor genistein. Therefore, these results indicate that the drug-induced activation of PTKs, which can be inhibited by genistein, was the initial and prerequisite signal leading to FasL upregulation and apoptosis, and preceded the activation of ERK2 and JNK1. Since it has been reported that the TCR activation of PTKs in T cells is able to activate MAP kinase family members such as ERK1/ERK2 through a Ras or PKC activation pathway and JNK through low-molecular-weight GTP-binding proteins, in particular Rac and Cdc42 [17, 27, 39], the activation of PKC in Jurkat T cells following aburatubolactam C-treatment was investigated by examining the translocation of PKC from the cytosol to the particulate membrane fraction. Although cPKCs, such as PKCα, βI, and βII were abundant and existed primarily in the cytosol, among the seven different PKC isoforms detected in the Jurkat T cells, neither the cPKC isoforms nor nPKCθ was translocated to the membrane fraction within 30 min after treatment with aburatubolactam C. However, translocation of nPKCδ and ε, and aPKCμ to the particulate membrane fraction was detected, along with a decrease in their levels in the cytosolic fraction, suggesting that nPKCs (δ and ε) and aPKCμ were involved in linking the aburatubolactam C-induced PTK activation signal to the downstream regulator ERK2. The aburatubolactam C-induced PKC modulation is significantly different from that previously observed in PMA-stimulated Jurkat T cells, as PMA is able to induce a rapid and apparent translocation of cPKCα and β and nPKCε, but with no detectable translocation of nPKCδ [35], suggesting that the selective activation of PKC isoforms may be critical for determining the fate of Jurkat T cells, as regards the proliferation or induction of FasL upregulation and apoptosis. To better understand whether the aburatubolactam C-induced ERK2 activation occurred via PKC activation and was critical for the induced FasL upregulation and apoptotic DNA fragmentation, the effect of PKC modulators, such as the PKC inhibitor GF109203X [19] and PKC activator PMA, on the drug-induced apoptotic DNA fragmentation and cellular changes, including the FasL upregulation and MAP kinase activation, were investigated. It was interesting to note that the drug-induced apoptotic DNA fragmentation, FasL upregulation, and JNK activation appeared to be augmented by GF109203X. Both tyrosine phosphorylation of multiple cellular proteins and the typical electrophoretic mobility shift of $p56^{lck}$ to $p60^{lck}$ induced by aburatubolactam C were also detected in the presence of GF109203X. However, the drug-induced ERK2 activation was completely blocked by GF109203X. Therefore, these results demonstrated that the aburatubolactam C-induced ERK2 activation dictated by the PKC signaling pathway negatively affected the drug-induced FasL upregulation and thus the development of apoptotic DNA fragmentation. Furthermore, the drug-induced activation of JNK was not apparently mediated by PKC activation and played a critical role in the FasL upregulation and Fas death signaling. The negative function of the PKC/ERK2 pathway in the aburatubolactam C-induced FasL expression was confirmed based on the observation that PMA was able to diminish the aburatubolactam C-induced apoptotic DNA fragmentation and FasL upregulation with a significant enhancement of ERK2 activation. Although the regulatory effect of PKC activation on apoptotic cell death depending on the cell type and apoptotic stimuli remains controversial, several reports have shown that the anti-apoptotic signaling of PKC is mediated by ERK1/2 [28, 33, 42]. In addition, it has been shown that JNK activation contributes to anisomycin-induced apoptosis in Jurkat T cells [11] and to 5-fluorouracil-induced apoptosis of hepatocellular carcinoma cells [9] by upregulating FasL expression. Consequently, the present results extend the previous findings and suggest a concerted role for the PTK/PKC/ERK2 and PTK/JNK1 pathways in drug-induced FasL expression and apoptosis. To obtain direct evidence that the FasL/Fas system is responsible for the aburatubolactam C-mediated apoptosis, two experiments were conducted. Firstly, the cytotoxic effect of aburatubolactam

C on Jurkat T cells was tested in the presence of the anti-Fas neutralizing antibody ZB-4. Secondly, the effect of the anti-apoptotic protein Bcl-2, which is known to protect cells from FasL/Fas interaction-mediated apoptosis, on the cytotoxicity of aburatubolactam C was also investigated. The cytotoxicity of aburatubolactam C toward the Jurkat T cells was not completely abrogated by either ZB-4 or ectopically overexpressed Bcl-2, but a reduction was detectable in both cases, confirming the contribution of the FasL/Fas system to the aburatubolactam C-induced apoptosis of Jurkat T cells. These results also suggested that the FasL upregulation and subsequent Fas-mediated apoptosis were not the only systems involved in the aburatubolactam C-induced cell death. Under these experimental conditions, the Fas-mediated cytotoxicity provoked by Fas-agonistic antibody CH-11 was almost completely blocked by either ZB-4 or overexpressed Bcl-2, demonstrating that both ZB-4 and overexpressed Bcl-2 are functionally active. Several studies have reported that Fas and/or FasL can be upregulated in tumor cells by antineoplastic drugs, and the cells subsequently undergo apoptosis in a process similar to activation-induced T cell death. However, the transcriptional mechanisms by which Fas and/or FasL can be upregulated in response to chemotherapeutic agents remain largely unknown. A recent study has shown that the JNK activation pathway and transcription factor AP-1 composed of the Jun-Fos heterodimer are required for FasL upregulation in hepatoma cells upon treatment with antineoplastic drugs [9]. Although the molecular mechanism was not explored further in the current study, the significant aburatubolactam C-mediated enhancement in the expression level of c-Fos mRNA, in accordance with JNK1 activation and FasL upregulation in the Jurkat T cells, seemed to indicate the possible involvement of the activation of AP-1 through the upregulation of c-Fos expression, as well as JNK1 activation in the aburatubolactam C-induced FasL upregulation in the Jurkat T cells.

In conclusion, the present results demonstrated that the apoptosis induced in Jurkat T cells by the novel PTK activator aburatubolactam C was exerted by FasL upregulation, dictated by the direct activation of PTKs ($p56^{lck}$ and $p59^{fyn}$), and subsequent induction of the JNK1 pathway, which could be promoted or attenuated by the inhibition or activation of the PKC/ERK2 pathway proximal to the PTK activation. Furthermore, the present results also indicated that aburatubolactam C possesses a potency applicable to the chemotherapeutic treatment of malignant cells.

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