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A Novel β-Lactam Antibiotic Activates Tumor Cell Apoptotic Program by Inducing DNA Damage

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ABSTRACT

Many of the anticancer drugs in current use are toxic and thus limited in their efficacy. It therefore becomes essential to develop novel chemotherapeutic agents with lower levels of toxicity. The β-lactam antibiotics have been used for many years to treat bacterial infections with limited or no toxicity. Until now, it has never been shown that β-lactams could kill tumor cells. Here, for the first time, we have discovered and characterized the apoptosis-inducing properties of a family of novel β-lactam antibiotics against human leukemia, breast, prostate, and head-and-neck cancer cells. We found that one particular lead compound (lactam 1) with an *N*-methylthio group was able to induce DNA damage and inhibit DNA replication in Jurkat T cells within a 2-h treatment. This was followed by p38 mitogen-activated protein kinase activation, S phase arrest, and apoptotic cell death. p38 was found to be a central player in β-lactam-induced apoptosis and resided downstream of DNA damage but upstream of caspase activation. Accompanying caspase-8 activation was cleavage of the pro-apoptotic Bcl-2 family protein Bid, and release of the mitochondrial cytochrome *c*. This was also associated with activation of caspase-9 and -3. Analogs of lactam 1 in which the *N*-methylthio group was replaced with other organothio chains exhibited progressive decreased potencies to induce DNA damage, p38 kinase activation, S phase arrest, and apoptosis, demonstrating requirement of the *N*-methylthio group. Because of the ease of synthesis and structural manipulation, we believe these β-lactams may have the potential to be developed into anticancer agents.

Apoptosis is the process by which a cell will actively commit suicide through a tightly controlled program (Wyllie et al., 1980). Morphologically, apoptosis is characterized by shrinkage of the cell, dramatic reorganization of the nucleus, active membrane blebbing, and, ultimately, fragmentation of the cell into membrane-enclosed vesicles (apoptotic bodies) (Earnshaw, 1995). Apoptosis occurs in two physiological stages, commitment and execution (Earnshaw, 1995).

Recent experiments have demonstrated that mitochondria play an essential role in apoptotic commitment (Green and Reed, 1998). Upon apoptotic stimulation, several important events occur at the mitochondria, including the release of cytochrome *c*. Release of cytochrome *c* can be inhibited by the expression of antiapoptotic Bcl-2 family members (such as Bcl-2 and Bcl-X_L) and induced by the expression of proapoptotic Bcl-2 family proteins (such as Bax and Bid) (Green and Reed, 1998). During receptor-mediated apoptosis, Bid is cleaved at its N terminus by caspase-8. The carboxyl-terminal fragment of Bid (molecular mass, 15 kDa) is then inserted into the membrane of the mitochondria, triggering release of mitochondrial cytochrome *c* (Li et al., 1998).

Once cytochrome *c* is released from the mitochondria, this commits the cell to die by either apoptosis or necrosis (Green and Reed, 1998). The cytochrome *c*-induced apoptotic process involves Apaf-1–mediated caspase activation. This cytosolic cytochrome *c* interacts with Apaf-1, which induces its asso-

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ABBREVIATIONS: Apaf-1, apoptotic protease-activating factor 1; PARP, poly(ADP-ribose) polymerase; MAP, mitogen-activated protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Ab, antibody; COX, cytochrome oxidase unit II; TUNEL, terminal deoxynucleotidyl transferase-mediated UTP nick-end labeling; Z-IETD-AFC, *N*-benzyloxycarbonyl-Ile-Glu-Thr-Asp-7-amino-4-trifluoromethyl coumarin; Ac-LEHD-AFC, *N*-acetyl-Leu-Glu-His-Asp-7-amino-4-trifluoromethyl coumarin; Ac-DEVD-AMC, *N*-acetyl-Asp-Glu-Val-Asp-amino-4-methylcoumarin; Ac-IETD-CHO, *N*-acetyl-Ile-Glu-Thr-Asp-CHO (aldehyde); Z-LE(OMe)HD(OMe)-FMK, *N*-benzyloxycarbonyl-Leu-Glu(OMe)-His-Asp(OMe)-fluoromethyl ketone; Ac-DEVD-CHO, *N*-acetyl-Asp-Glu-Val-Asp-CHO (aldehyde); Boc-D-FMK, *N*-*tert*-butoxycarbonyl-Asp-fluoromethyl ketone; PD169316, 4-(4-fluorophenyl)-2-(4-nitrophenyl)-5-(4-pyridyl)-1H-imidazole; PBS, phosphate-buffered saline; TdT, terminal deoxynucleotidyl transferase; SAR, structure-activity relationship; DMSO, dimethyl sulfoxide; MT-21, a previously reported synthetic γ lactam.

ciation with procaspase-9, thereby triggering processing and consequent activation of caspase-9. The activated caspase-9 in turn cleaves downstream effector caspases (such as caspase-3), initiating apoptotic execution (Green and Reed, 1998). It is thought that the activation of effector caspases leads to apoptosis through the proteolytic cleavage of important cellular proteins, such as poly(ADP-ribose) polymerase (PARP) (Lazebnik et al., 1994) and the retinoblastoma protein (An and Dou, 1996; Janicke et al., 1996).

Activation of the cellular apoptotic program is a current strategy for the treatment of human cancer. It has been demonstrated that radiation and standard chemotherapeutic drugs kill some tumor cells through induction of apoptosis (Fisher, 1994). Unfortunately, the majority of human cancers at present are resistant to these therapies (Desoize, 1994; Harrison, 1995). It is therefore essential to identify novel apoptosis-inducing compounds that are candidate antitumor agents. Along this line, synthetic small molecules have great potential to be developed into anticancer drugs because they can be easily synthesized and structurally manipulated for selective development.

For more than 60 years, the β-lactam antibiotics have played an essential role in treating bacterial infections (Lukacs and Ohno, 1990). Traditional β-lactam antibiotics do not affect eukaryotic cells and are nontoxic to human cell lines. Recently, a new class of *N*-thiolated β-lactams was found to inhibit bacterial growth in *Staphylococcus aureus* (Ren et al., 1998; Turos et al., 2000). Until this study, no research had shown that a β-lactam antibiotic could have anticancer activities. Our interest in both β-lactams (Ren et al., 1998; Turos et al., 2000) and anticancer drug discovery (An et al., 1998; Dou and Nam, 2000) prompted our current study. Here we report, for the first time, that β-lactam derivatives (Fig. 1) rapidly induce DNA damage, inhibit DNA replication, and activate the apoptotic death program in human leukemic Jurkat T cells, in a time- and concentration-dependent manner. Lactam 1 (Fig. 1) also inhibits proliferation and induces apoptosis in other human solid tumor cell lines such as breast, prostate, and head-and-neck. Induction of apoptosis by lactam 1 is associated with activation of p38 mitogen-activated protein (MAP) kinase, release of mitochondrial cytochrome *c*, and activation of the caspases. Apoptosis is blocked by a specific inhibitor to p38 kinase, implicating p38 MAP kinase as a central player in β-lactam-induced apoptosis.

Experimental Procedures

Materials. Fetal calf serum, propidium iodide, MTT, trypan blue, and RNase A were purchased from Sigma-Aldrich (St. Louis, MO). RPMI 1640 medium, Dulbecco's modified Eagle's medium, penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA). Polyclonal antibodies to human PARP were obtained from Roche Molecular Biochemicals (Indianapolis, IN); polyclonal antibodies to caspase-8 (Ab-1) were obtained from Oncogene Research Products (Boston, MA). Monoclonal antibodies to Tyr-182-phosphorylated and total p38 protein were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies to caspase-9 (Ab-2) and caspase-3 (Ab-1) were from Oncogene Research Products; antibodies to cytochrome *c* were from BD PharMingen (San Diego, CA);and antibodies to cytochrome oxidase unit II (COX) were from Molecular Probes (Eugene, OR). Goat antibody to actin and anti-rabbit IgG-horseradish peroxidase were obtained from Santa Cruz Biotechnology. The APO-DIRECT kit for terminal deoxynucleotidyl transferase-mediated UTP nick-end labeling (TUNEL) staining was purchased from BD PharMingen. [*methyl*-^{3}H]Thymidine was obtained from Amersham Biosciences (Piscataway, NJ). Z-IETD-AFC (the specific caspase-8 substrate), Ac-LEHD-AFC (the specific caspase-9 substrate), Ac-DEVD-AMC (the specific caspase-3 substrate), Ac-IETD-CHO (the specific caspase-8 inhibitor), Z-LE(OMe)HD(OMe)-FMK (the specific caspase-9 inhibitor), Ac-DEVD-CHO (the specific caspase-3 inhibitor), Boc-D-FMK (a pan-caspase inhibitor), and PD169316 (the specific p38 MAP kinase inhibitor) were obtained from Calbiochem (San Diego, CA).

Synthesis of β-Lactams. β-Lactams 1 to 7 (Fig. 1) were prepared as racemates (with *cis* stereochemistry) using a procedure described previously (Ren et al., 1998; Turos et al., 2000). Full experimental details and spectral data will be published separately.

Cell Cultures, Protein Extraction, and Western Blot Assay. Human Jurkat T cells were cultured in RPMI 1640 medium, supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Human breast cancer MCF7 and MDA-MB-231 cells, human prostate cancer PC-3 cells, and human head-and-neck cancer PCI-13 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, penicillin, and streptomycin. All the cell lines were maintained in a 5% CO_2 atmosphere at 37°C. A whole-cell extract was prepared as described previously (An et al., 1998). Briefly, cells were harvested, washed with PBS, and homogenized in a lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol) for 30 min at 4°C. After that, the lysates were centrifuged at 14,000*g* for 30 min, and the supernatants were collected as whole-cell extracts. Equal amounts of protein extract (50 μg) were resolved by SDS-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH) using a Semi-Dry Transfer System (Bio-Rad,

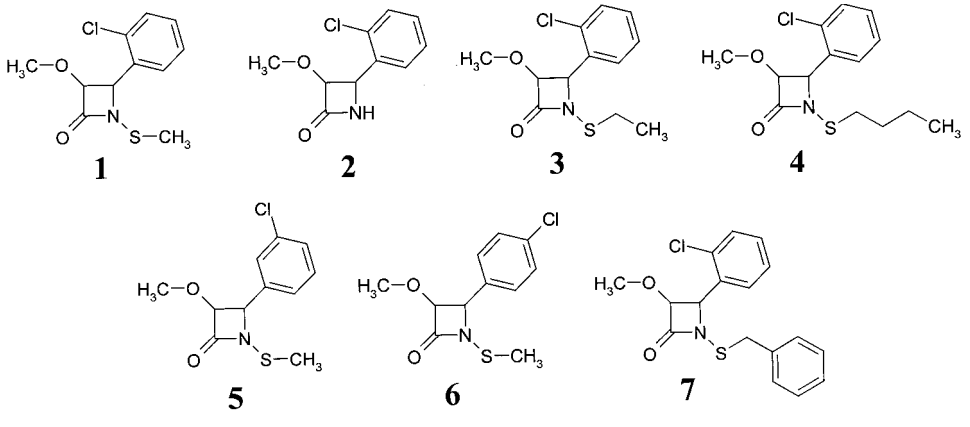


Fig. 1. Structures of seven representative β-lactams.

Hercules, CA). The enhanced chemiluminescence Western blot analysis was then performed using specific antibodies to the proteins of interest.

Cell-Free Caspase Activity Assay. Cell-free caspase activities were determined by measuring the cleavage of amino-4-methylcoumarin or 7-amino-4-trifluoromethyl coumarin groups from each respective caspase substrate, as we described previously (Nam et al., 2001) with some modifications. Briefly, a prepared protein extract (20 μg) was incubated in a buffer containing 50 mM Tris/pH 8.0 along with each respective caspase substrate at 20 μM in a 96-well plate. The reaction mixture was incubated at 37°C for 2 h. After incubation, the liberated fluorescent amino-4-methylcoumarin or 7-amino-4-trifluoromethyl coumarin groups were measured by a Wallac Victor^2 1420 Multilabel counter (Wallac Victor, Turku, Finland) with 355/460 nm and 405/535 nm filters, respectively.

Trypan Blue Assay. The trypan blue exclusion assay was done by injecting 10 μl of cell suspension containing 0.2% trypan blue dye into a hemocytometer and counting. Numbers of cells that absorbed the dye and those that excluded the dye were counted, from which the percentage of nonviable cell number to total cell number was calculated.

Subcellular Fractionation. Both cytosolic and mitochondrial fractions were isolated at 4°C using a previous protocol (Gao and Dou, 2000) with some modifications. At each time point, cells were washed twice with PBS, resuspended in a hypotonic buffer containing 20 mM HEPES, pH 7.5, 1.5 mM $MgCl_2$, 5 mM KCl, and 1 mM dithiothreitol, and incubated on ice for 10 min. The cells were lysed 30 times in a Dounce homogenizer, and the lysate was centrifuged at 2,000*g* for 10 min. The supernatant was collected and centrifuged again at the same condition. The resulting supernatant was then centrifuged at 20,500*g* for 30 min, followed by collection of both the supernatant (cytosol) and pellet fractions. The pellet was washed twice with a buffer containing 210 mM mannitol, 70 mM sucrose, 5 mM Tris-HCl, pH 7.5, and 1 mM EDTA, and resuspended in the lysis buffer as the mitochondrial fraction.

Flow Cytometry. Cell cycle analysis based on DNA content was performed as we described previously (An et al., 1998). At each time point, cells were harvested, counted, and washed twice with PBS. Cells (5×10^6) were suspended in 0.5 ml of PBS, fixed in 5 ml of 70% ethanol for at least 2 h at −20°C, centrifuged, resuspended again in 1 ml of propidium iodide staining solution (50 μg of propidium iodide, 100 units of RNase A, and 1 mg of glucose per ml of PBS), and incubated at room temperature for 30 min. The cells were then analyzed with FACScan (BD Biosciences, San Jose, CA), ModFit LT and WinMDI V.2.8 cell cycle analysis software (Verity Software, Topsham, ME). The cell cycle distribution is shown as the percentage of cells containing G_1, S, G_2, and M DNA judged by propidium iodide staining. The apoptotic population is determined as the percentage of cells with sub-G_1 ($<G_1$) DNA content.

[^{3}H]Thymidine Incorporation Assay. Incorporation of [^{3}H]thymidine into cells was measured by a previous protocol (Smith and Dou, 2001). Jurkat T cells were pretreated with a selected lactam for the indicated number of hours, followed by coincubation with 2 μl/ml [*methyl*-^{3}H]thymidine [80 Ci (1.5 TBq)/mmol] at 37°C for 2 h. After harvesting, the cell pellet was washed with PBS, resuspended in 0.5 ml of PBS, and collected on a glass microfiber filter. The filter was then washed with 5 ml/filter of PBS, followed by 5 ml/filter of ice-cold 0.1N NaOH and 5 ml/filter of ethanol. The filters containing fixed DNA were dried, and the remaining radioactivity was measured on a scintillation counter (Smith and Dou, 2001).

TUNEL Assay. TUNEL was performed to determine the extent of DNA strand breaks (Smith and Dou, 2001). TUNEL assay was performed with an APO-Direct kit per the manufacturer's instructions. In brief, cells were fixed in 1% paraformaldehyde and ethanol at 20°C overnight and then permeabilized with Proteinase K. After permeabilization, fluorescein-conjugated dNTPs and terminal deoxynucleotidyl transferase (TdT) were added to the cells. TdT was then able to label free ends of DNA with fluorescein-conjugated dNTPs that could then be detected by flow cytometry. For the fluorescence microscopy of TUNEL-positive cells, Jurkat T cells were labeled and analyzed per the manufacturer's instructions and our previous method (An et al., 1998).

MTT Assay. MCF7, MDA-MB-231, PC-3, and PCI-13 cells were grown to 50% confluence in a 24-well plate. Triplicate wells of cells were then treated with 50 μM lactam 1 for 24 h. A stock 5 mg/ml MTT in serum-free medium was then added to the cell cultures at a final concentration of 1 mg/ml, followed by a 3-h incubation at 37°C. After cells were crystallized, the medium was removed and DMSO was added to dissolve the metabolized MTT product. The absorbance was then measured on a Wallac Victor^2 1420 Multilabel counter at 540 nm.

Nuclear Staining Assay. To assay nuclear morphology, the detached or remaining attached solid tumor cells were washed with PBS, fixed with 70% ethanol for 1 h, and stained with Hoechst 33342 (50 μM) for 30 min. The nuclear morphology of cells was visualized by a fluorescence microscope (An et al., 1998).

Results

Screen for Apoptotically Active β-Lactams. A library of β-lactam analogs was screened for their ability to induce apoptosis. A representative group of seven compounds and their structures is shown in Fig. 1. The screening procedure was accomplished by treating human Jurkat T cells with each compound at 50 μM for 8 h. This was followed by preparation of cell lysates and measurement of apoptosis-specific caspase-3 activation (by cell-free caspase-3 activity assay) and PARP cleavage (by Western blotting).

Among the tested compounds, lactam 1 was found to have the greatest potency to induce caspase-3 activation and PARP cleavage within 8 h of treatment (Fig. 2, A and B). Several important structure-activity relationships (SARs) were observed. First and most significantly, the *N*-methylthio group is required for the apoptosis-inducing activity of lactam 1. Lactam 2, which is an analog of lactam 1 that lacks

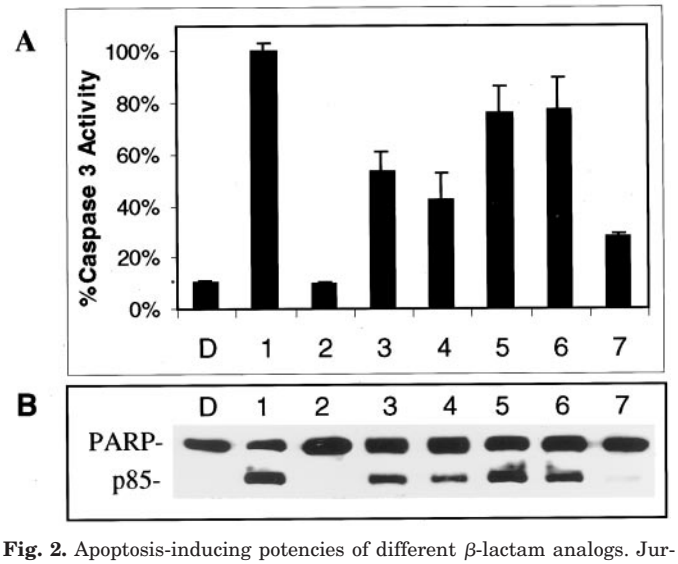


Fig. 2. Apoptosis-inducing potencies of different β-lactam analogs. Jurkat T cells were treated with either a 50 μM concentration of each individual compound or the vehicle DMSO (indicated by D) for 8 h, followed by preparation of cellular extracts. A, cell-free caspase-3 activity assay. Standard deviations were calculated from five separate and independent experiments and are indicated by error bars. B, Western blot assay using a specific polyclonal PARP antibody. The intact PARP (molecular mass, 116 kDa) and a PARP cleavage fragment (p85) are shown. Similar results were obtained in four or more experiments.

the *N*-methylthio group (Fig. 1), induced neither caspase-3 activation nor PARP cleavage (Fig. 2, A and B, 2 versus 1). In fact, lactam 2-treated cells showed no morphological changes, similar to that observed for DMSO (vehicle)-treated cells (Fig. 2, A and B, 2 versus D, and data not shown).

The second SAR observed was that an increase in the number of carbons on the *N*-thio group was inversely proportional to the apoptosis-inducing ability of these β-lactams. An increase from one carbon (lactam 1) to two carbons (lactam 3) in this chain decreased ~50% of caspase-3 activity and PARP cleavage (Fig. 2, A and B, 1 versus 3). A further increase to four carbons on the *N*-thio group (lactam 4) caused ~65% decrease in the apoptosis-inducing activity (Fig. 2, A and B, 4 versus 1). Replacement of the *N*-methylthio with an *N*-benzylthio group (Fig. 1, lactam 7) also decreased the apoptosis-inducing activity by ~70% (Fig. 2, A and B, 1 versus 7).

Another SAR was found for the chlorophenyl group in lactam 1. Lactams 1, 5, and 6 are isomers with the chlorine group at *ortho*-, *meta*-, and *para*-positions, respectively, on the phenyl ring (Fig. 1). Although lactams 5 and 6 both had similar potency in inducing caspase-3 activity and PARP cleavage, they were less potent than lactam 1 (by ~20%; Fig. 2, A and B). Based on these results, we chose lactam 1 as a lead compound for further apoptosis and cell cycle studies.

Lactam 1-Induced Apoptosis Is Caspase-Dependent and Associated with Cytochrome *c* Release. We studied the lactam 1-induced apoptosis further by performing both kinetics and concentration-response experiments. When Jurkat T cells were treated with 50 μM lactam 1 for 2, 4, 6, 8, 12, or 24 h, apoptosis occurred in a time-dependent manner (Fig. 3, A and B). The PARP cleavage fragment p85 appeared after 4 h of treatment, and its levels increased afterward (Fig. 3A). Associated with this, the nonviable cell population, as determined by a trypan blue exclusion assay, was increased by 20% at 4 h, which was further increased to 60% after 24-h treatment of lactam 1 (Fig. 3B).

To determine which caspases are activated during lactam 1-induced apoptosis, we measured activation of caspase-8, -9, and -3 by both cell-free activity assay (Fig. 3C) and Western blot analysis (Fig. 3D). The caspase-8 activity was detected at 2 h and later time points, with a maximal level at 6 h (Fig. 3C). Western blot assay confirmed cleavage and activation of caspase-8 at 2 h with peaking amounts of caspase-8 fragment at 6 h (molecular mass, 18 kDa; Fig. 3D). Consistent with caspase-8 activation, a 15-kDa fragment of Bid (Li et al., 1998) was observed as early as 2 h after lactam 1 treatment and peaked at 6 h (Fig. 3D). The activity of caspase-9 was first detected at 4 h and then increased afterward (Fig. 3C). The increased level of the caspase-9 activity was associated with increased levels of the active caspase-9 fragment (molecular mass, 35 kDa; Fig. 3D). However, caspase-9 activity levels (by enzyme activity assay) and cleavage fragment amounts (by Western blot) were lower than those detected for caspase-8 (Fig. 3, C and D). The cell-free caspase-3 activity was also observed first at 4 h and dramatically increased after 6 h of treatment (Fig. 3C), with kinetics very similar to that of caspase-3 cleavage detected by Western blotting (Fig. 3D). Furthermore, kinetically, caspase-3 activation was parallel to PARP cleavage (Fig. 3, C and D versus A), which agrees with the observation that caspase-3 is responsible for cleaving PARP (Lazebnik et al., 1994). Therefore, lactam 1-induced apoptosis is associated with activation of these caspases.

It has been shown that MT-21, a synthetic compound with a γ-lactam ring (a class of structures different from the β-lactams studied here), was able to induce mitochondrial cytochrome *c* release and apoptotic cell death (Watabe et al., 2000). We then determined whether lactam 1 was able to induce cytochrome *c* release from the mitochondria. In an experiment similar to that in Fig. 3, Jurkat T cells were treated with lactam 1 for up to 12 h, followed by isolation of cytosolic and mitochondrial fractions and measurement of the cytochrome *c* levels (Fig. 4). High levels of mitochondrial cytochrome *c* were detected in untreated cells, associated with low levels of cytosolic cytochrome *c* (Fig. 4A). After 2 to 4 h of treatment with lactam 1, levels of mitochondrial cytochrome *c* were decreased, whereas those of cytosolic cytochrome *c* were significantly increased (Fig. 4A), indicating release of cytochrome *c* from the mitochondria. Although the mitochondrial cytochrome *c* levels were further decreased after 6-h or longer treatment, little or no cytochrome *c* was detected in the cytosol (Fig. 4A), suggesting loss of cytosolic cytochrome *c* in the later stages of apoptosis (compare with Fig. 3B). The observed cytochrome *c* release from mitochon-

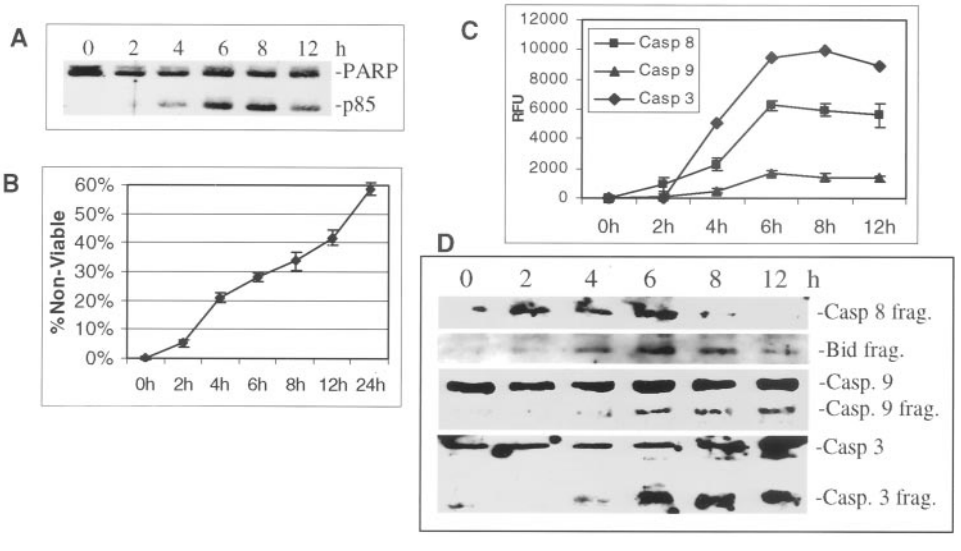


Fig. 3. A kinetic characterization of lactam 1-induced apoptosis. Jurkat T cells were treated with 50 μM lactam 1 for the indicated hours, followed by performance of various assays as follows. A and D, Western blot assay using specific antibodies to PARP, caspase-8, Bid, caspase-9, or caspase-3. The proenzyme and the active cleavage fragment of caspase-9 (46 and 35 kDa, respectively) and caspase-3 (32 and 17 kDa, respectively), as well as the active fragment of caspase-8 (18 kDa) and Bid (15 kDa), are shown. The experiments were done a minimum of three times with similar results. B, trypan blue incorporation assay. The numbers given are percentages of nonviable cells to total cells. Standard deviations are shown with error bars from a mean of at least three different experiments. C, fluorogenic cell-free caspase-8, -9, and -3 assay. Standard deviations are shown from three independent experiments.

dria to the cytosol was not an artifact, because we also observed constitutive levels of the mitochondria-specific COX (Fig. 4B) and the cytosolic β-actin protein (Fig. 4C). Importantly, release of cytochrome *c* began before activation of caspase-3 (Fig. 4 versus Fig. 3).

We then treated Jurkat T cells for 8 h with various concentrations of lactam 1 (Fig. 5). Induction of apoptosis-specific PARP cleavage was dependent on the concentrations of lactam 1 used. Low levels of p85 PARP fragment were detected when 20 μM lactam 1 was used, which were further increased at 30 and 40 μM and significantly increased at 50 μM. At 60 μM, lactam 1 caused almost complete degradation of both the intact PARP protein and the p85 PARP fragment (Fig. 5A). We also found that loss of membrane permeability, a late event in apoptosis (Wyllie et al., 1980; Earnshaw, 1995), was also lactam 1-concentration-dependent: ~10% at 20 to 40 μM, ~30% at 50 μM, and ~80% at 60 μM (Fig. 5B).

When cell-free caspase activity assay was performed in this experiment, activation of caspase-8, -9, and -3 was also found to depend on concentrations of lactam 1 (Fig. 5C). Compared with lysates of untreated cells (0 μM), the levels of caspase-8 were increased by 2-fold when 30 to 40 μM lactam 1 was used and by 5-fold when 50 μM lactam 1 was used (Fig. 5C). Levels of caspase-3 were increased by 2-, 3-, 5-, and 11-fold, respectively, when lactam 1 was used at 20, 30, 40, or 50 μM (Fig. 5C). Higher levels of caspase-9 activity were also detected in lysates of cells treated with higher concentrations of lactam 1, although caspase-9 activity levels were again lower than those detected for caspase-8 and -3 (Fig. 5C).

Both caspase activation and apoptosis induction were observed in time- and concentration-dependent fashions (Figs. 3 and 5), supporting the hypothesis that caspases are required for lactam 1-induced apoptotic cell death. To further examine this hypothesis, Jurkat T cells were pretreated for 1 h with an individual caspase inhibitor, a general caspase inhibitor (pan), or the vehicle (DMSO), followed by a cotreatment for 8 h with 50 μM lactam 1. Pre- and coincubation with each of the used caspase inhibitors completely blocked lactam 1-induced PARP cleavage (Fig. 5D) and apoptosis-associated morphological changes (data not shown). Therefore, activation of the caspases is required for lactam 1-induced apoptosis.

Lactam 1-Induced Apoptosis Is Associated with an Increased S Phase Population. It has been suggested that dysregulation of cell cycle progression is involved in the initiation of apoptosis (Lee et al., 1993; Dou, 1997; Smith et al., 2000). To determine whether lactam 1-induced apoptosis is associated with cell cycle-specific changes, we measured the cell cycle distribution of Jurkat T cells that had been treated with lactam 1 in the same kinetics (Fig. 3) and concentration-response (Fig. 5) experiments.

In the kinetics experiment, a slight decrease in G_1 and a corresponding increase in S phase population (2–3%) were first observed after lactam 1 treatment for 2 to 4 h (Fig. 6A). This was accompanied by induction of apoptotic cell death, as measured by increased apoptotic sub-G_1 ($<G_1$) cell population (2%; Fig. 6A) and PARP cleavage (Fig. 3A). After 6 to 12 h of treatment, S phase population was further increased by up to 13%, whereas that of G_1 further decreased, without any apparent change in G_2/M population (Fig. 6A). Increased levels of sub-G_1 population (5–16%; Fig. 6A) and PARP cleavage (Fig. 3A) were also observed. A 24-h treatment with lactam 1 further increased S (20%) and sub-G_1 (30%) populations (Fig. 6A). These data suggest that lactam 1-induced apoptosis is associated with increased S phase population. This conclusion was further supported by results from the concentration-response experiment (data not shown).

While screening apoptotically active β-lactams, we found that compounds 2, 4, 3, and 1 increased cellular apoptosis in a stepwise fashion (Fig. 2). We then determined whether these lactams also caused S phase accumulation in a similar manner. Treatment of Jurkat T cells with 50 μM lactam 2 for 5 h did not induce accumulation of either S or sub-G_1 populations, similar to that of DMSO-treated cells (Fig. 6B, 2 versus D). In contrast, under the same conditions, treatment of lactams 4, 3, and 1 increased S phase population by 8, 15, and 21%, respectively (Fig. 6B), associated with stepwise increased sub-G_1 apoptotic populations, 2, 10, and 14%, respectively (Fig. 6B). These data suggest that the number of carbons bound to the *N*-thio group is important not only for its apoptosis-inducing activity but also for its ability to arrest cells in S phase.

Lactam 1 Inhibits DNA Replication, Associated with Induction of DNA Damage. To determine whether the increased S phase population by lactam 1 is caused by inhibition of DNA replication, a [^{3}H]thymidine incorporation as-

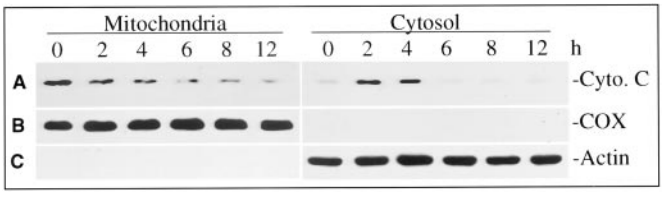


Fig. 4. Lactam 1 induces cytochrome *c* release from mitochondria. Cytosolic and mitochondrial fractions were prepared from Jurkat T cells treated with 50 μM lactam 1 for the indicated hours, followed by Western blot assay using a specific antibody to cytochrome *c* (molecular mass, 17 kDa; A), the cytochrome oxidase subunit II (COX, molecular mass, 26 kDa; B), and β-actin (molecular mass, 43 kDa; C). The unchanged levels of mitochondrial COX and cytosolic β-actin protein serve as controls for equal loading and fractionation purity.

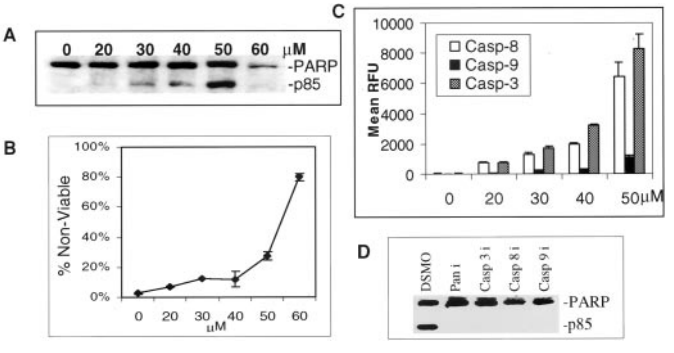
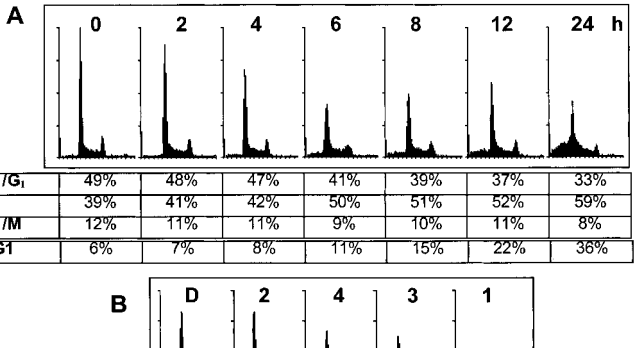


Fig. 5. A to C, concentration-dependent characterization of apoptosis induced by lactam 1. Jurkat T cells were treated with increasing concentrations of lactam 1 for 8 h, followed by assaying PARP cleavage (A), trypan blue incorporation (B), and cell-free caspase-8, -9, and -3 activities (C), as described in the legend to Fig. 3. Results were representative of three to five different experiments. Standard deviations are given with error bars from a mean of at least three different experiments in B and C. D, caspase inhibitors block apoptosis induced by lactam 1. Jurkat T cells were pretreated with either an inhibitor to caspase-8, -9, or -3 (at 25 μM), or a general caspase inhibitor (pan, at 25 μM), or DMSO, followed by a cotreatment with 50 μM lactam 1 for 8 h. After that, PARP cleavage was determined in a Western blot assay. RFU, relative fluorescence units, measured by released fluorescent 7-amino-4-methylcoumarin from substrate.

say was performed with or without lactam 1 in both kinetics and concentration-response experiments. In the kinetics experiment, Jurkat T cells were pretreated with 50 μM lactam 1 or DMSO for 0, 2, 4, 6, or 8 h, followed by a 2-h cotreatment with [^{3}H]thymidine. After that, cells were harvested and the amount of incorporated radioactive [^{3}H]thymidine was determined. When both lactam 1 and [^{3}H]thymidine were added at the same time and then coincubated for 2 h, incorporation of the [^{3}H]thymidine was inhibited by ~70%, compared with the control cells (Fig. 7A, 0 h versus C). A preincubation with lactam 1 for 2 to 8 h caused 95% inhibition of [^{3}H]thymidine incorporation. Thus, lactam 1 is able to inhibit [^{3}H]thymidine incorporation, and does so immediately after its administration. The fact that lactam 1 inhibits [^{3}H]thymidine incorporation within such a short time period (2 h) argues that lactam 1 is directly affecting the ability of the cell to replicate its DNA, and this effect is not due to a change in cell cycle (see Fig. 6A).

In the concentration-response experiment, Jurkat T cells were preincubated for 2 h with various concentrations of lactam 1, followed by a 2-h coincubation with [^{3}H]thymidine (a total treatment length of 4 h). Inhibition of [^{3}H]thymidine incorporation was found to depend on lactam 1 concentrations used: 20% inhibition at 20 μM, 45% at 30 μM, 90% at 40 μM, and ~100% at 50 or 60 μM. The half-maximal inhibition value for incorporation of [^{3}H]thymidine (IC_{50}) in intact Jurkat cells was determined to be 32 μM.

We hypothesized that lactam 1 could induce DNA damage that would lead to the inhibition of DNA replication observed (Fig. 7, A and B), which would then be responsible for blockage of S phase progression (Fig. 6) and induction of apoptosis (Figs. 1–6; see also Fig. 10). To test this hypothesis, we implemented a TUNEL assay that detects DNA strand breaks, and the TUNEL-positive cells were either quantified by flow cytometry or observed under fluorescence microscopy.



	0 h	2 h	4 h	6 h	8 h	12 h	24 h
G_0/G_1	49%	48%	47%	41%	39%	37%	33%
S	39%	41%	42%	50%	51%	52%	59%
G_2/M	12%	11%	11%	9%	10%	11%	8%
<G1	6%	7%	8%	11%	15%	22%	36%

	D	2	4	3	1
G_0/G_1	50%	51%	45%	38%	35%
S	28%	29%	36%	43%	49%
G_2/M	22%	20%	19%	19%	16%
<G1	1%	1%	3%	11%	15%

Fig. 6. Lactam 1 dysregulates cell cycle progression that is associated with apoptosis induction. A, Jurkat T cells were treated with 50 μM lactam 1 for the indicated hours, followed by flow cytometry analysis. The cell cycle distribution was measured as the percentage of cells that contain G_1, S, G_2, and M DNA (cells in G_1, S, G_2, and M = 100%). The apoptotic population was measured as the percentage of total cell populations with $<G_1$ DNA content. B, Jurkat T cells were treated with either DMSO (D) or 50 μM lactam 2, 4, 3, or 1 for 5 h, followed by assaying the cell cycle distribution and sub-G_1 population, as described in A. Similar results were obtained in at least three other independent experiments.

Treatment with lactam 1 for 1 h did not induce DNA strand breaks, compared with the untreated cells (0 h) that stained negative for nick-end labeling (Fig. 7C, 0 h versus 1 h). However, after just 2 h incubation with lactam 1, more than half of the cell population had shifted into the M_1 region, which demonstrated a positive signal for DNA strand breaks (Fig. 7C, 2 h). At this time, the S phase population was only slightly increased (compare Fig. 6A), and apoptosis had not been initiated (Fig. 3A). After 4 h of treatment with lactam 1, almost the entire population of Jurkat cells contained damaged DNA, as shown by both flow cytometry (Fig. 7C) and fluorescence microscopy (Fig. 7D). Under the same conditions, the S population slightly increased (Fig. 6A), and apoptosis just started to be detectable (Fig. 3A). These results suggest that lactam 1 induces DNA strand breaks before S phase accumulation and apoptosis induction. The fact that lactam 1 induces DNA damage in the entire cell population within 4 h (Fig. 7, C and D) also indicates that it acts via a cell cycle-independent manner.

We then tested whether the order of potencies of lactams 2, 4, 3, and 1 to induce apoptosis (Figs. 2 and 6) and S phase accumulation (Fig. 6) matched that of their DNA-damaging abilities. After 4 h of treatment, DMSO or lactam 2 did not cause any DNA damage, whereas lactams 4 and 3 induced DNA damage in 3 and 16% of the cell population, respectively (Fig. 7E). Again, treatment with lactam 1 for 4 h caused nearly 100% of the cell population to become TUNEL-positive (Fig. 7E). As a comparison, the traditional DNA-damaging agent etoposide (VP-16) at the same concentration induced only 10% of the cells to become TUNEL-positive (Fig. 7E). Therefore, the DNA-damaging abilities of these lactams, which are inversely proportional to the number of carbons on the *N*-thio constituent, match exactly their potencies to induce cell cycle dysregulation and apoptosis.

p38 MAP Kinase Activation Is Necessary for Lactam 1-Induced Apoptosis. It has been shown that multiple stimuli, including DNA-damaging agents, induce apoptosis via activation of p38 MAP kinase (Kummer et al., 1997; Sanchez-Prieto et al., 2000). Because lactam 1 was able to induce DNA strand breaks (Fig. 7, C and D), we then examined whether lactam 1 could activate p38 MAP kinase during apoptosis induction. In this experiment, Jurkat T cells were treated with 50 μM lactam 1 for up to 12 h, followed by measuring levels of phosphorylated (the activated) and total p38 protein in Western blot assay. The levels of Tyr-182-phosphorylated p38 protein were increased by 3-fold at 2 h and reached maximum (~9-fold) by 6 h (Fig. 8A). It has been shown that dual phosphorylation of p38 on Tyr-182 and Thr-180 activates this kinase (Raingeaud et al., 1995). In contrast, the levels of total p38 protein remained relatively unchanged (Fig. 8B). Therefore, it seems that lactam 1-induced DNA damage triggers activation of p38 before S population accumulation and apoptosis induction.

Because lactams 2, 4, 3, and 1 exhibited a sequential increase in DNA-damaging activities (Fig. 7E), we predicted that these compounds should also have the same order of potencies to induce p38 phosphorylation. Indeed, lactam 2 was unable to increase the levels of phosphorylated p38 after a 4-h treatment (Fig. 8D). In contrast, lactam 4 shows a 2.6-fold normalized increase in phosphorylation of p38 over that of the control (Fig. 8D). Lactam 3 shows a further activation of p38 with a 3.2-fold induction over the control.

Again, lactam 1 induces maximal p38 phosphorylation with a 5.9-fold increase (Fig. 8D). Therefore, an increase in the number of carbons on the *N*-thio constituent of the lactams causes a stepwise decrease in the ability of these compounds to induce DNA damage, p38 phosphorylation, S phase accumulation, and apoptosis.

To determine whether p38 activation is necessary for the apoptotic effects elicited by lactam 1, Jurkat T cells were pretreated with either PD169316, a specific p38 kinase inhibitor (Kummer et al., 1997), or the vehicle DMSO for 1 h, followed by a cotreatment with lactam 1 for 8 h. Pre- and cotreatment with PD169316 completely inhibited the process of PARP cleavage induced by lactam 1, compared with the control cells (Fig. 8E). In addition, PD-169316 potently inhibited lactam 1-induced activation of caspase-8, -9 and -3, as measured by cell-free caspase activity assay (Fig. 8F). In fact, the caspase-inhibitory effects of the p38 kinase inhibitor were comparable with those of the pan-caspase inhibitor (Fig. 8F). Because these results were so striking, we investigated whether PD169316 could potentially inhibit caspase activity directly. Jurkat T cells were treated with 50 μM VP-16 for 5 h, followed by preparation of cell lysates and measurement of cell-free caspase activities, in the absence or presence of PD169316 or the pan-caspase inhibitor. We found that PD169316 could not inhibit caspase-8, -9, or -3 activities in the VP-16-treated cell preparation. In contrast, the pan-caspase inhibitor completely blocked the VP-16-induced caspase activities (data not shown). This result suggests that during lactam 1-induced apoptosis, p38 activation occurs upstream of caspase activation and is necessary for caspase-mediated cell death.

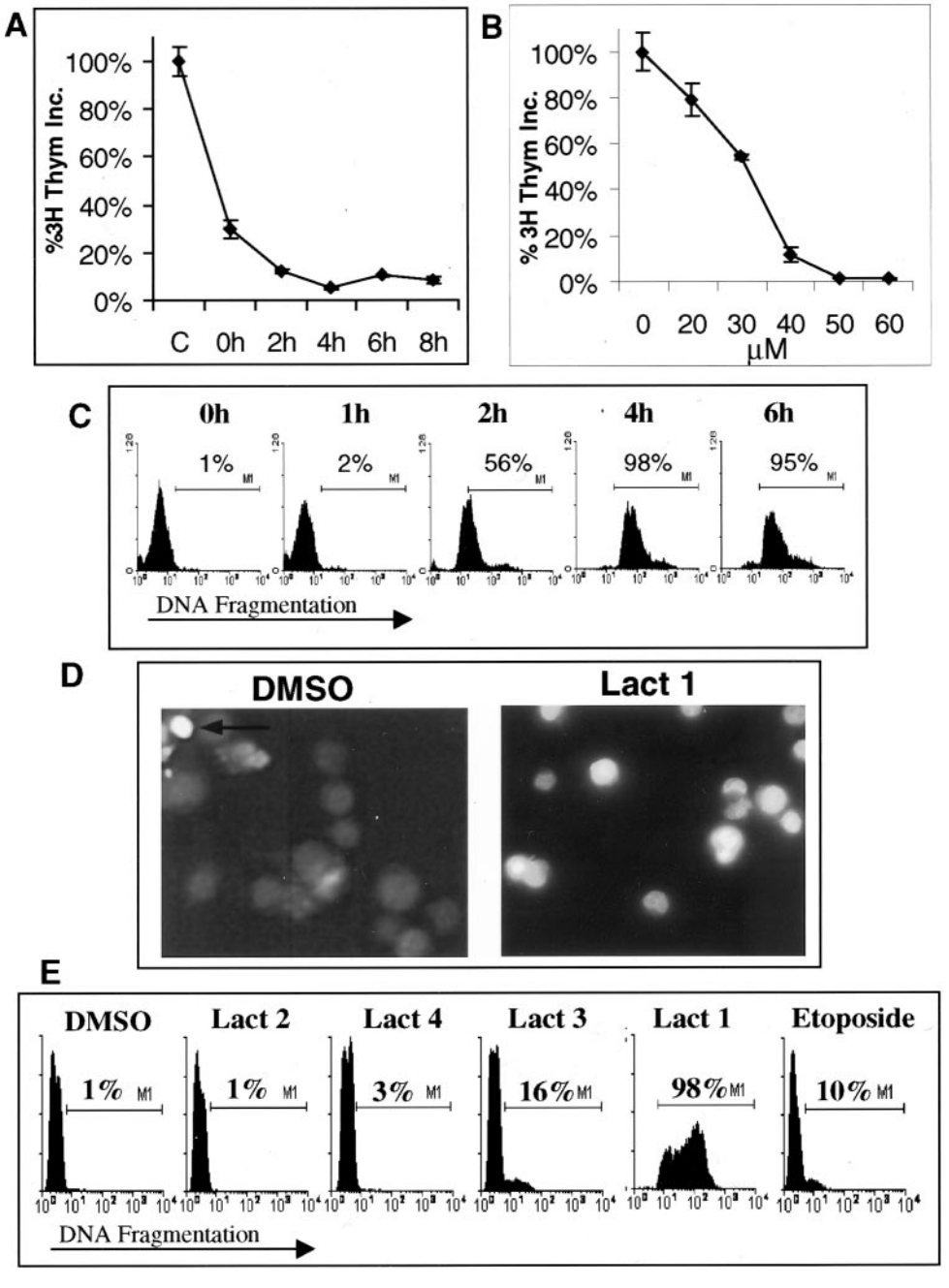


Fig. 7. Lactam 1 inhibits DNA replication and induces DNA strand breaks in Jurkat T cells. A and B, [^{3}H]thymidine incorporation assay. Jurkat T cells were either untreated (as a control, indicated by C or 0 μM), or pretreated with 50 μM lactam 1 for the indicated hours (A), or pretreated with the indicated concentrations of lactam 1 for 2 h (B). After that, [^{3}H]thymidine was added, followed by an additional 2-h incubation. The amount of [^{3}H]thymidine incorporated was then analyzed by scintillation counting (see *Experimental Procedures*). Standard deviations are shown with error bars from a mean of at least three different experiments. C to E, TUNEL assay. Jurkat cells (0 h) were treated with 50 μM lactam 1 for 4 h (D) or at each indicated time point (C), or with a 50 μM concentration of the indicated drug for 4 h (E), followed by analysis of DNA strand breaks by flow cytometry (C and E) or fluorescence microscopy (D). The M1 region represents the TUNEL-positive (DNA strand break) cells. Similar results were observed in three independent experiments.

Given that lactam 1 is able to inhibit DNA replication (Fig. 7) and induce apoptosis (Figs. 1–6) and that lactam 1-induced apoptosis can be blocked by PD169316 (Fig. 8, E and F), we then determined whether the p38 inhibitor could affect the DNA replication-inhibitory activity of lactam 1. To do so, a [^{3}H]thymidine incorporation assay was performed using Jurkat cells treated with lactam 1 alone or a combination of lactam 1 and PD169316. Inhibition of DNA replication by lactam 1 was not affected by addition of PD169316 (Fig. 8G). Thus, the p38 kinase inhibitor only inhibits the lactam 1-induced downstream apoptotic events but does not affect the ability of lactam 1 to inhibit DNA replication.

To further investigate the order of lactam 1-induced apoptotic events, we measured effects of PD-169316 and the pan-caspase inhibitor Boc-D-FMK on TUNEL positivity and p38 phosphorylation. In this experiment, growing Jurkat T cells (control) were treated for 4 h with lactam 1 in the absence (with DMSO) or presence of PD169316 or Boc-D-FMK, followed by measurement of TUNEL-positive cells and phosphorylated p38 levels. Again, lactam 1 treatment induced 96% TUNEL positivity. Similarly, cells that had been cotreated with lactam 1 and PD169316 or Boc-D-FMK showed 95% and 97% TUNEL positivity, respectively (Fig. 8H), demonstrating that neither the p38 inhibitor nor the pan-caspase inhibitor could block DNA strand breaks induced by lactam 1. These data also suggest that DNA damage must lie upstream of p38 and caspase activation. In addition, lactam 1-induced p38 phosphorylation was not affected by Boc-D-FMK (Fig. 8I), supporting the conclusion that p38 activation occurs upstream of caspase activation (compare with Fig. 8F).

Lactam 1 Inhibits Cell Proliferation and Induces Apoptosis in Several Solid Tumor Cell Lines. After we determined that lactam 1 could inhibit cell cycle progression (Fig. 6) and induce apoptosis (Figs. 1–6) in leukemia Jurkat T cells, we studied effects of this compound on several other human solid tumor cell lines. Exponentially grown (0 h) human breast (MCF7, MDA-MB-231), prostate (PC-3), and head-and-neck (PCI-13) cancer cell lines were treated with either 50 μM lactam 1 or DMSO for 24 h, followed by performance of an MTT assay, which measures the status of cell viability and, thus, cell proliferation. The DMSO-treated cells continued to proliferate after 24 h (Fig. 9A). However, after treatment with lactam 1, cellular viability of MCF7, MDA-MB-231, and PCI-13 cells was decreased by 80% and that of PC-3 cells decreased by 60% (Fig. 9A).

To determine whether lactam 1-mediated growth inhibition is caused by cell death, these tumor cell lines were treated with 50 μM lactam 1 or an equal percentage of DMSO, followed by separation of the attached and detached cell populations. Both attached and detached cell populations were then used for detection of apoptotic nuclear changes. We found that after a 24-h treatment with lactam 1, ~50% of MCF7 cells became detached. All the detached MCF7 cells exhibited typical apoptotic nuclear condensation and fragmentation (Fig. 9B). The cellular detachment is most likely triggered by apoptosis induction, because the remaining attached MCF7 cells also showed apoptotic nuclear morphology (Fig. 9B). Little or no detachment was observed in MCF7 cells treated with DMSO; consistent with that, all the remaining attached cells contained normal, round nuclei (Fig. 9B). Similar to MCF7 cells, about half of the MDA-MB-231, PC-3, and PCI-13 cells became detached after a 48-h treatment with lactam 1 but not DMSO. Almost all the detached cells exhibited an apoptosis-specific nuclear morphology (Fig. 9B). These data demonstrate that lactam 1 is able to inhibit cell proliferation and induce cell death in these breast, prostate, and head-and-neck solid tumor cell lines.

Discussion

An important property of a candidate anticancer drug is the ability to induce tumor cell apoptosis (Fisher, 1994). Toward the goal of developing novel chemotherapeutic agents, in this current study, we have examined whether β-lactams have apoptosis-inducing abilities. We found that

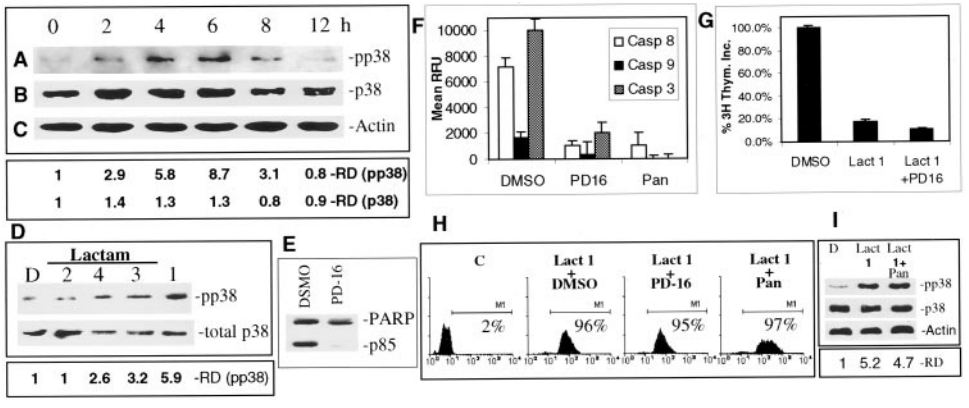


Fig. 8. Involvement of p38 MAP kinase in lactam 1-induced apoptosis. A–D, Jurkat T cells were treated with 50 μM lactam 1 for the indicated hours (A–C) or with 50 μM lactam 2, 4, 3, or 1 for 5 h (D), followed by Western blot assay using specific antibodies to phosphorylated p38 (pp38), total p38 (p38), or actin. Relative density (RD) values are normalized ratios of the intensities of the pp38 or p38 band to the corresponding actin band. The experiments were done three times with similar results. E and F, Jurkat T cells were pretreated for 1 h with either the specific p38 MAP kinase inhibitor PD169316 (PD-16; at 30 μM), the pan-caspase inhibitor (at 25 μM), or the vehicle DMSO, followed by a cotreatment with 50 μM lactam 1 for 8 h. After that, PARP cleavage was determined in Western blotting (E), and caspase-8, -9, and -3 activities were measured in cell-free assay (F; see Fig. 3). G, Jurkat T cells were pretreated for 2 h with either DMSO, lactam 1 (at 50 μM), or lactam 1 (50 μM) plus PD169316 (30 μM), followed by addition of [^{3}H]thymidine. After an additional 2-h incubation, the amount of [^{3}H]thymidine incorporated was then analyzed by scintillation counting (see Fig. 7). H and I, Jurkat T cells were treated for 4 h with either DMSO or lactam 1 (at 50 μM), in the absence (with DMSO) or presence of PD169316 (PD-16; at 30 μM) or Boc-D-FMK (50 μM), followed by measurement of TUNEL positivity and p38 phosphorylation as described in Figs. 7C and 8, A to C, respectively.

lactam 1, the most potent β-lactam selected in this study, was able to induce apoptosis in human leukemic (Jurkat T), breast (MCF7, MDA-MB-231), prostate (PC-3), and head-and-neck (PCI-13) cancer cell lines. Lactam 1-induced apoptosis was caspase-dependent, associated with cytochrome *c* release. In addition, lactam 1-induced apoptosis was preceded by induction of DNA damage, activation of p38 kinase, and inhibition of S phase progression. Studies using specific inhibitors demonstrated the requirement of p38 kinase for lactam 1-induced, caspase-dependent cell death. Finally, the order of the potencies of several structurally different β-lactams to induce DNA damage matched well with their potencies to activate p38 kinase, inhibit S phase progression, and induce apoptosis.

Our results indicate that lactam 1 induces apoptosis via activation of caspases (Figs. 3 and 5). As demonstrated by both Western blot and enzyme activity assay, caspase-8 activity was increased after just 2 h of treatment with lactam 1, consistent with the appearance of the active form of the Bid cleavage fragment (Fig. 3). Caspase-9 and -3 were found to be cleaved and activated subsequently or at the same times (Fig. 3). It has been shown that caspase-8 is able to cleave and thus activate other executionary caspases such as caspase-3 both directly and indirectly as in feedback loops (Green and Reed, 1998). One such feedback loop is the Bid-caspase-9 pathway. Caspase-8 can cleave Bid, allowing it to translocate to the mitochondria and release cytochrome *c* into the cytosol. The release of cytochrome *c* can promote apoptosome formation and activation of caspase-9, which in turn cleaves and activates caspase-3 (Green and Reed, 1998). Consistent with this pathway, lactam 1 treatment was able to induce mitochondrial cytochrome *c* release along with caspase activation (Figs. 4 and 10).

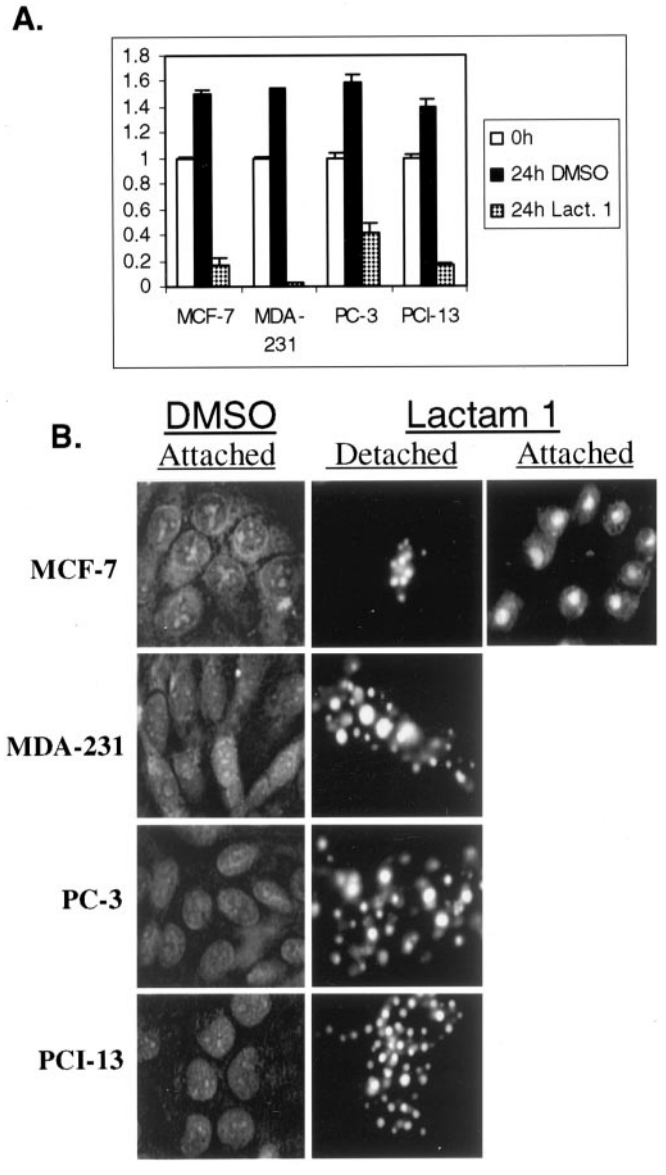


Fig. 9. Lactam 1 inhibits proliferation and induces apoptosis in four solid tumor cell lines. A, MTT assay. Human breast (MCF7, MDA-MB-231), prostate (PC-3), and head-and-neck (PCI-13) cancer cell lines were grown in equal cell numbers in a 24-well plate. At ~50% confluence (0 h), three wells of each cell line were treated with either 50 μM lactam 1 or DMSO for 24 h. After that, cells were subjected to MTT assay (see *Experimental Procedures*). Standard deviations are given as described in Fig. 3. B, nuclear staining assay. MCF7, MDA-MB-231, PC-3, and PCI-13 cells were treated with 50 μM lactam 1 or DMSO for 24 (MCF7) or 48 h (the other three cell lines), followed by collection of both detached and attached cell populations. After lactam 1 treatment, ~50% of cells of these cancer lines became detached, whereas <5% were detached from the treatment with DMSO. Both detached and attached cell populations were used for nuclear staining assay with DNA staining dye Hoechst 33342. Each sample was then analyzed by fluorescence microscopy for nuclear morphology. Similar results were obtained in six independent experiments.

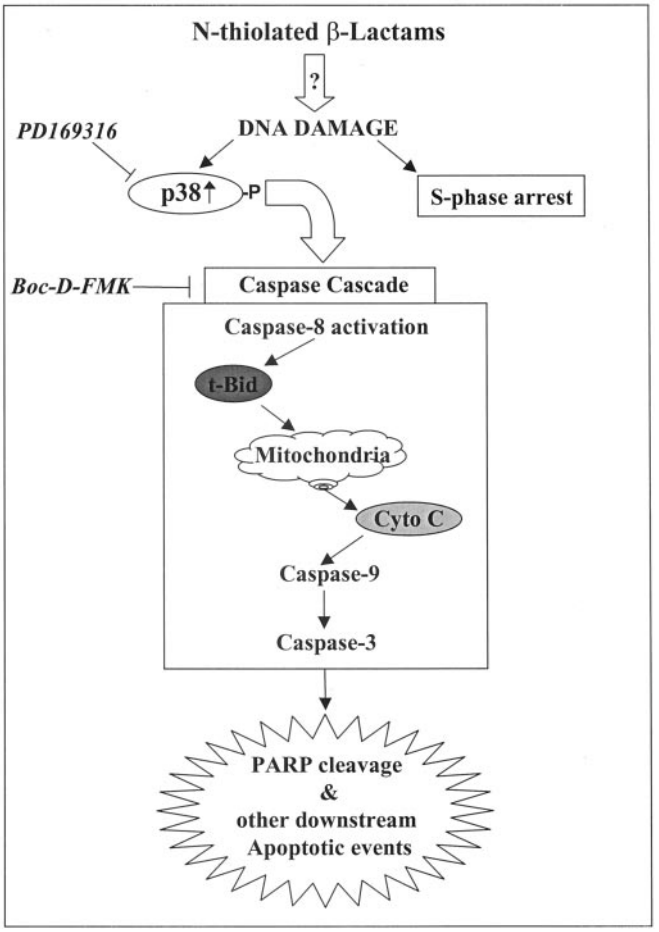


Fig. 10. The proposed order of apoptotic events induced by lactam 1. A cascade of events that occur during lactam 1-induced apoptosis is proposed based on kinetic and inhibitor studies. See *Results* for details.

Many traditional pharmacological agents induce cell death in a cell cycle-dependent manner, whereas others do not (Meikrantz et al., 1994; Dou, 1997; Orren et al., 1997; Smith et al., 2000). We have found that lactam 1 at 50 μM induces an S phase arrest, associated with apoptosis induction (Fig. 6). This arrest in S phase was attributed to inhibition of DNA replication as shown by a [^{3}H]thymidine incorporation assay (Fig. 7). [^{3}H]Thymidine incorporation (a measure of DNA replication) was inhibited by lactam 1 treatment nearly immediately after administration (Fig. 7A). Inhibition of DNA replication can be caused by multiple mechanisms, including DNA damage. Indeed, results from TUNEL assay showed that nearly 100% of the cell population contained DNA stand breaks after just 4 h of incubation with lactam 1 (Fig. 7C). However, at this time, there was still no appearance of sub-G_1 cells (Fig. 6A), suggesting that the apoptosis-associated DNA fragmentation had not yet occurred. Our data strongly suggest that lactam 1 has the ability to induce DNA strand breaks. Several traditional chemotherapeutic drugs such as topoisomerase inhibitors and other DNA-damaging agents also cause DNA strand breaks in this fashion (Kohlhagen et al., 1998; Tronov et al., 1999). We tested lactam 1 in a topoisomerase II concatenation assay and found that lactam 1 did not inhibit topoisomerase II activity (data not shown). In addition, in a plasmid relaxation assay, it seemed that lactam 1 did not directly cause cleavage of DNA (data not shown). The mechanism by which lactam 1 induces DNA strand breaks, therefore, remains elusive. However, we can conclude that lactam 1 induces DNA strand breaks at as early as 2 h and can also inhibit the incorporation of [^{3}H]thymidine within 2 h, before any cell cycle change has taken place. As expected, these mechanisms also result in S phase arrest and apoptosis (Fig. 10).

p38 MAP kinase is a stress-response kinase that is bifurcate and can regulate both cell proliferation and apoptosis (Birkenkamp et al., 1999). It has been shown that p38 causes the up-regulation of death receptors and ligands such as Fas (Hsu et al., 1999) and TNF-α (Brinkman et al., 1999), which are activators of caspase-8. Traditional chemotherapeutic modalities such as VP-16 and cisplatin have also been shown to induce apoptosis through genotoxic stresses, which cause activation of p38 (Kummer et al., 1997; Ono and Han, 2000; Sanchez-Prieto et al., 2000). After determining that lactam 1 caused damage to DNA, we also found that p38 MAP kinase was an essential mediator of apoptosis in response to DNA damage induced by lactam 1 (Fig. 8). p38 was activated after just 2 h of lactam 1 treatment (Fig. 8A), which also correlates kinetically with the time that DNA strand breaks are first introduced (Fig. 7C). It seems that activation of p38 by lactam 1 was essential for induction of apoptosis as shown by utilization of a specific p38 inhibitor, which completely blocked apoptosis induced by lactam 1 (Fig. 8E). Therefore, transduction of the apoptotic signal by p38 must also lie downstream of DNA damage, which was also supported by failure of PD169316 to inhibit TUNEL positivity (Fig. 8H). This suggests that lactam 1 induces DNA strand breaks that cause genomic stresses and, therefore, p38 activation, which in turn activates downstream signals for apoptosis initiation (Fig. 10).

Our results indicate that p38 activation occurs upstream of and also is probably required for caspase activation. Indeed, when cells were coincubated with lactam 1 and Boc-D-FMK, p38 phosphorylation and DNA damage were not affected (Fig. 8, H and I; see also Fig. 10). This led us to question the role of p38 activation in this cascade. Figure 8F demonstrated that PD169316 could inhibit lactam 1-induced caspase activation, supporting the conclusion that p38 lies upstream of the caspases and p38 activity is needed to elicit a caspase activation induced by lactam 1.

The most important SAR observed in the current study came from a comparison among lactams 2, 3, 4, and 1 (Fig. 1). The rank of potencies of these four lactams to induce DNA damage (Fig. 7E) matches precisely the order for activation of p38 MAP kinase (Fig. 8D), inhibition of S phase progression (Fig. 6B), and induction of apoptosis (Figs. 2 and 6B). Therefore, the ability to damage DNA in tumor cells is essential for the apoptosis-inducing activity of β-lactams, and these activities require the presence of the *N*-methylthio moiety. It is possible that transfer of the *N*-methylthio moiety is necessary for the observed biological activities. One of our future studies will examine the chemical basis of action of these *N*-thiolated β-lactams and the molecular target of β-lactams in cancer cells.

One of the important criteria for potential anticancer drugs is the ability to selectively kill tumor, but not normal, cells. Our preliminary data suggested that lactam 1 was able to selectively induce apoptosis in simian virus 40-transformed, but not the parental normal, human fibroblasts (data not shown). Another future focus for our studies will be to systematically compare the effects of these β-lactams on both tumor and normal cell lines and investigate the involved molecular mechanisms.

Large amounts of work and research are currently being performed on compounds that show apoptosis-inducing activity. There are even a few widely used anticancer drugs for which the mechanisms of action are not yet fully understood. Although the direct target of lactam 1 is unknown at this moment, our current studies have indicated that lactam 1 has great potential as a lead compound that could be developed into a novel anticancer drug.

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