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Novel *N*-thiolated β-lactam antibiotics selectively induce apoptosis in human tumor and transformed, but not normal or nontransformed, cells

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Abstract

Historically, it has been shown that the β-lactam antibiotics play an essential role in treating bacterial infections while demonstrating selectivity for prokaryotic cells. We recently reported that certain *N*-methylthio-substituted β-lactam antibiotics had DNA-damaging and apoptosis-inducing activities in various tumor cells. However, whether these compounds affect human normal or nontransformed cells was unknown. In the current study, we first show that a lead compound (lactam **1**) selectively induces apoptosis in human leukemic Jurkat T, but not in the nontransformed, immortalized human natural killer (NK) cells. Additionally, we screened a library of other *N*-methylthiolated β-lactams to determine their structure–activity relationships (SARs), and found lactam **12** to have the highest apoptosis-inducing activity against human leukemic Jurkat T cells, associated with increased DNA-damaging potency. Furthermore, we demonstrate that lactam **12**, as well as lactam **1**, potently inhibits colony formation of human prostate cancer cells. We also show that lactam **12** induces apoptosis in human breast, prostate, and head-and-neck cancer cells. Finally, lactam **12** induces apoptosis selectively in Jurkat T and simian virus 40-transformed, but not in nontransformed NK and parental normal fibroblast, cells. Our results suggest that there is potential for developing this class of β-lactams into novel anticancer agents.
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Keywords: *N*-thiolated β-lactam; Antibiotics; DNA damage; Apoptosis; Anticancer drugs

1. Introduction

Apoptosis, or programmed cell death, is a highly regulated process important in embryonic and immune system development and tissue homeostasis [1,2]. Perturbation of this pathway can lead to autoimmunity, acquired immune deficiency syndrome, neurodegenerative disorders, and cancer [3,4]. Initiation, commitment, and execution are the three fundamental steps of apoptosis [5]. Several apoptotic stimuli, such as death receptor-binding ligands, signal to activate the initiator caspases (caspases-2, -8, -9, -10), which in turn activates downstream effector caspases (caspases-3, -6, -7). The effector caspases can also be activated through the release of key mitochondrial proteins, such as cytochrome *c*, cell death inducer second mitochondria-derived activator of caspases (Smac), and apoptosis initiating factor [6]. It is generally believed that proteolytic cleavage of a variety of intracellular substrates, including poly(ADP-ribose) polymerase (PARP) [7,8] and the retinoblastoma protein (RB) [9–11], by effector caspases leads to apoptosis.

For nearly 60 years β-lactam compounds have been used in the treatment of bacterial infections [12]. Following the initial introduction of penicillin, a variety of other classes of β-lactam antibiotics were subsequently identified and used clinically, including cephalosporins, penems,

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Abbreviations: SAR, structure–activity relationship; PARP, poly(ADP-ribose) polymerase; TUNEL, terminal deoxynucleotidyl transferase-mediated UTP nick-end labeling; NK cells, natural killer cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide.

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carbapenems, nocardicins, and monobactams [13]. The bacterial targets of these antibiotics are membrane-bound transpeptidases referred to as the penicillin-binding proteins, which are responsible for creating crosslinks within the bacterial cell wall [13]. By disrupting these crosslinking proteins, the β-lactams induce structural deformities within the cell wall, which cause the bacteria to lyse. Recently, a novel class of *N*-thiolated β-lactams has been shown to inhibit *Staphylococcus aureus* and methicillin-resistant *S. aureus* growth [14–16].

Previously we showed that *N*-thiolated β-lactams, such as β-lactam **1**, induced DNA damage, inhibited DNA replication, and induced tumor cell apoptosis in a time- and concentration-dependent manner [17]. Our current study shows, for the first time, that the *N*-thiolated β-lactam **1** can preferentially induce apoptosis in leukemic Jurkat T cells, but not nontransformed, immortalized human NK cells. Additionally, we also show that lactam **12**, an analog of lactam **1**, has enhanced apoptosis-inducing activity in Jurkat T cells compared to lactam **1**. Furthermore, this study reveals that lactam **12** can induce apoptosis in other human solid tumor cell lines such as breast, prostate, and head and neck. Lactam **12** also induces apoptosis selectively in Jurkat T, but not human NK, cells, and in simian virus 40 (SV40)-transformed human fibroblasts (VA-13), but not in their parental counterpart (WI-38). Both lactams **1** and **12** are able to activate caspase-3 in human prostate cancer cells and inhibit colony formation of these cells in soft agar. These data indicate that further study of *N*-thiolated β-lactams in the treatment of cancer is warranted.

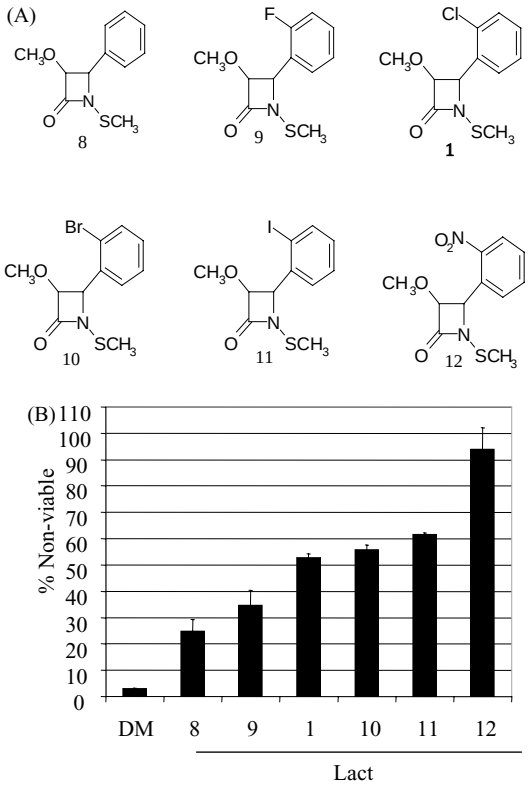

Fig. 1. Screen for more potent analogs of the lactam **1**. (A) Structures of the *N*-thiolated β-lactam compounds studied. Numerical designations were given to each compound. (B) Jurkat T cells were treated with the solvent (DMSO) or 50 μM of each indicated analog for 24 hr, followed by trypan blue dye exclusion 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.

2. Materials and methods

2.1. Materials

Fetal bovine serum (Tissue Culture Biologicals), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and trypan blue were purchased from Sigma-Aldrich. RPMI 1640, Dulbecco's modified Eagle's medium (DMEM), MEM nonessential amino acids solution, MEM sodium pyruvate solution, penicillin, and streptomycin were purchased from Invitrogen. Fluorogenic peptide substrate Ac-DEVD-AMC (the specific caspase-3/-7 substrate) was obtained from Calbiochem. Polyclonal antibody to human PARP was obtained from Roche Molecular Biochemicals. The APO-DIRECT kit for terminal deoxynucleotidyl transferase-mediated UTP nick-end labeling (TUNEL) staining was purchased from BD Pharmingen.

2.2. Synthesis of β-lactams

The β-lactam analogs (Fig. 1A) were prepared as racemates (with *cis* stereochemistry) using a procedure described previously [14,15].

2.3. Cell culture, protein extraction, and Western blot assay

Human Jurkat T cells and human prostate cancer LNCaP cells were cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Human YT cells were cultured in RPMI 1640 medium supplemented with 1 mM MEM sodium pyruvate solution, 0.1 mM MEM nonessential amino acids solution, 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Human breast cancer MCF-7 cells, head-and-neck cancer PCI-13 cells, prostate cancer DU-145 cells, normal (WI-38) and SV-40 transformed (VA-13) human fibroblasts cells were grown in DMEM containing 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. All cell lines were maintained at 37° in a humidified incubator with an atmosphere of 5% CO_2.

A whole-cell extract was prepared as described previously [18]. 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% NP-40, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol) for 30 min at 4°. Afterwards, the lysates were centrifuged

at 12,000 *g* for 15 min at 4° and the supernatants collected as whole-cell extracts. Equal amounts of protein extract (60 μg) were resolved by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Schleicher & Schuell) using a Semi-Dry Transfer System (Bio-Rad). The enhanced chemiluminescence Western blot analysis was then performed using specific antibodies to the proteins of interest.

2.4. Trypan blue assay

The trypan blue dye exclusion assay was performed by mixing 20 μL of cell suspension with 20 μL of 0.4% trypan blue dye before injecting into a hemocytometer and counting. The number of cells that absorbed the dye and those that exclude the dye were counted, from which the percentage of nonviable cell number to total cell number was calculated.

2.5. Morphological assessment of apoptosis

To assess morphological changes of cells, 50 μL of treated or untreated cell suspension were transferred to a glass slide at the indicated time points. The slides were observed under a phase-contrast microscope (Leica) and photographs were taken (100×). Apoptotic cells were identified by their distinct morphological changes.

2.6. TUNEL assay

Terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) was used to determine the extent of DNA strand breaks [19]. The assay was performed following manufacturer's instruction using the APO-Direct kit. In brief, the harvested cells were fixed in 1% paraformaldehyde for 15 min on ice, washed with PBS, and then fixed again in 70% ethanol at −20° overnight. The cells were then incubated in DNA labeling solution (containing terminal deoxynucleotidyl transferase (TdT) enzyme, fluorescein-conjugated dUTP and reaction buffer) for 90 min at 37°. After removing the DNA labeling solution by rinsing cells with Rinsing Buffer, the cells were incubated with the propidium iodide/RNase A solution, incubated for 30 min at room temperature in the dark, and then analyzed by flow cytometry within 3 hr of staining.

2.7. Caspase-3/-7 activity assay

To measure cell-free caspase-3/-7 activity, whole-cell extracts (20–30 μg) from untreated or treated LNCaP, MCF-7, PCI-13, DU-145, VA-13, and WI-38 cells were incubated with 20 μM of the fluorogenic substrate caspase-3/-7 (Ac-DEVD-AMC) for 30 min at 37° in 100 μL of assay buffer (50 mM Tris, pH 8.0). Measurement of the hydrolyzed AMC groups was performed on a VersaFluor™ Fluorometer (Bio-Rad) as described previously [20].

2.8. Soft agar assay

The soft agar assay was performed as described previously [21] with a few modifications. In brief, in a 6-well plate, a bottom feeder layer (0.6% agar) was prepared with DMEM media containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. A top layer (0.3% agar) was prepared with DMEM and the same media as described above but containing 2×10^4 prostate cancer LNCaP cells and 50 μM of lactam **1** or **12**, or equal volume of solvent (DMSO) as a control. Plates were incubated at 37° in 5% CO_2 in a humidified incubator for 3 weeks. MTT (1 mg/mL) was added to each well and incubated overnight to allow complete formation of purple formazan crystals. The plates were then scanned and photographed, and the number of colonies was quantified by *Quantity one* v. 4.0.3 software (Bio-Rad).

2.9. Nuclear staining

To assay nuclear morphology, the detached or remaining attached cells were washed with PBS, fixed with 70% ethanol for 1 hr, and stained with Hoechst 33342 (1 mM) for 30 min. The nuclear morphology was visualized by fluorescence microscopy (40×; Leitz) [18].

3. Results

3.1. Screening for more apoptotically active analogs of lactam *1*

Lactam **1** contains a *chloro* (–Cl) group in the *ortho* position on the benzene ring (Fig. 1A). To examine whether deletion or substitution of the Cl group could affect its cell death-inducing ability, other halogen and nonhalogen analogs of lactam **1** were synthesized (Fig. 1A). These compounds were then tested in the trypan blue dye exclusion assay, using lactam **1** as a comparison (Fig. 1B). Jurkat T cells were treated with each of these compounds at 50 μM for 24 hr, followed by measurement of loss of cell membrane permeability, indicative of a late apoptotic stage (Fig. 1B) [22,23]. As a control, lactam **1** induced ∼52% cell death (Fig. 1B). Interestingly, removal of the Cl group from the benzene ring significantly decreased the cell death-inducing activity to ∼25% (lactam **8**; Fig. 1B). Furthermore, replacement of the Cl group with a smaller halogen atom (–F; lactam **9**) also decreased the death-inducing activity (to ∼35%), while analogs with a larger halogen group (–Br and –I; lactams **10** and **11**, respectively; Fig. 1A) increased the cell death rates to 55 and 60% (Fig. 1B). These data suggest that the size of the group in the *ortho* position on the benzene ring is important for the compound's cell death-inducing activity. Indeed, the analog with $–NO_2$ substitution, lactam **12** (Fig. 1A), exhibited the strongest effect with a total of

~94% cell death (Fig. 1B). Therefore, the order of potency of the tested compounds was: X = H < F < Cl < Br < I < NO_2.

3.2. Lactam **1** induces apoptosis preferentially in leukemic Jurkat T over nontransformed, immortalized NK cells

Previously, we reported that β-lactam analogs, such as lactam **1** (Fig. 1A) [17], were able to induce tumor cell apoptosis. However, whether lactam **1** affects normal or nontransformed cells was unknown. To determine whether lactam **1** was able to induce apoptosis preferentially in tumor/transformed vs. normal/nontransformed cells, we treated human leukemic Jurkat T cells and immortalized, nontransformed NK cells (YT line) [24] with lactam **1** in both concentration- and time-dependent experiments. Treatment of Jurkat T cells with 10 μM of lactam **1** for 24 hr induced apoptosis-specific PARP cleavage fragment p85 (Fig. 2A), whose levels were further increased when 25 μM of lactam **1** was used (Fig. 2A). After treatment with 50 μM of lactam **1**, PARP degradation was further increased, as evidenced by a significant decrease in expression of intact PARP protein (Fig. 2A). In contrast, no PARP cleavage was detectable in the YT cells after treatment with lactam **1** at even 50 μM (Fig. 2A).

In the kinetic experiment, both Jurkat T and YT cells were treated with 30 μM of lactam **1** for 3, 6, or 24 hr. PARP cleavage was detected in Jurkat T cells first at 3 hr, which was then increased at 6 hr (although the levels of PARP/p85 fragments at 24 hr were decreased in this Western blotting; Fig. 2B). Importantly, no PARP cleavage was observed in YT cells in the same kinetics experiment (Fig. 2B). To confirm the tumor cell-selective killing activity of lactam **1**, a trypan blue dye exclusion assay was performed in the same kinetic experiment. After 24 hr, there was 42% cell death in the Jurkat T cells compared to 9% in YT cells (Fig. 2C). Furthermore, by using phase-contrast microscopy, more cell death was observed in Jurkat T cells than YT cells (Fig. 2D). These data support the conclusion that lactam **1** could induce apoptotic cell death selectively in tumor *over* nontransformed cells.

3.3. Lactam **12** has enhanced apoptosis-inducing activity specific to Jurkat T, but not normal YT cells

To determine if lactam **12** is capable of inducing apoptosis at lower concentrations than lactam **1**, a dose–response experiment was performed with both compounds. Jurkat T cells were treated with lactam **12** at 2, 10, 25, and 50 μM for 24 hr, using 50 μM of lactam **1** as a comparison. Again, treatment with lactam **1** caused ~50% cell death, measured by trypan blue exclusion assay (Fig. 3A). Under the same experimental conditions, lactam **12** induced cell death in a concentration-dependent manner: 25% at 10 μM, 45% at 25 μM, and 80–90% at 50 μM (Fig. 3A). Therefore, lactam **12** is ~2-fold more potent than lactam **1**. This conclusion was further supported by PARP cleavage

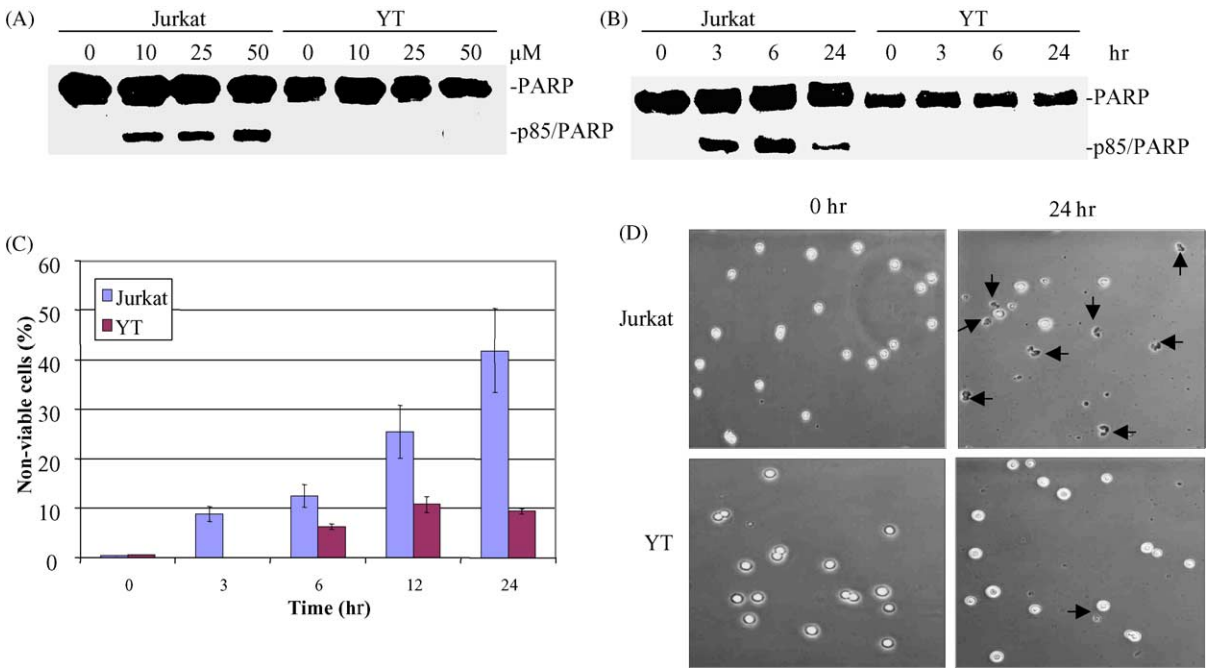

Fig. 2. Selective induction of apoptosis by lactam **1** in leukemic Jurkat T *over* immortalized/nontransformed NK cells. Jurkat T and NK (YT) cells were treated with 10, 25, and 50 μM of lactam **1** for 24 hr (A) or with 30 μM of lactam **1** for indicated hours (B–D). (A and B) Measurement of PARP cleavage in Western blot assay. The intact PARP (116 kDa) and a PARP cleavage fragment (p85) are shown. (C) Trypan blue dye exclusion 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. (D) Morphological changes of Jurkat T and YT cells after treatment. Photographs under a phase-contrast microscope (100×).

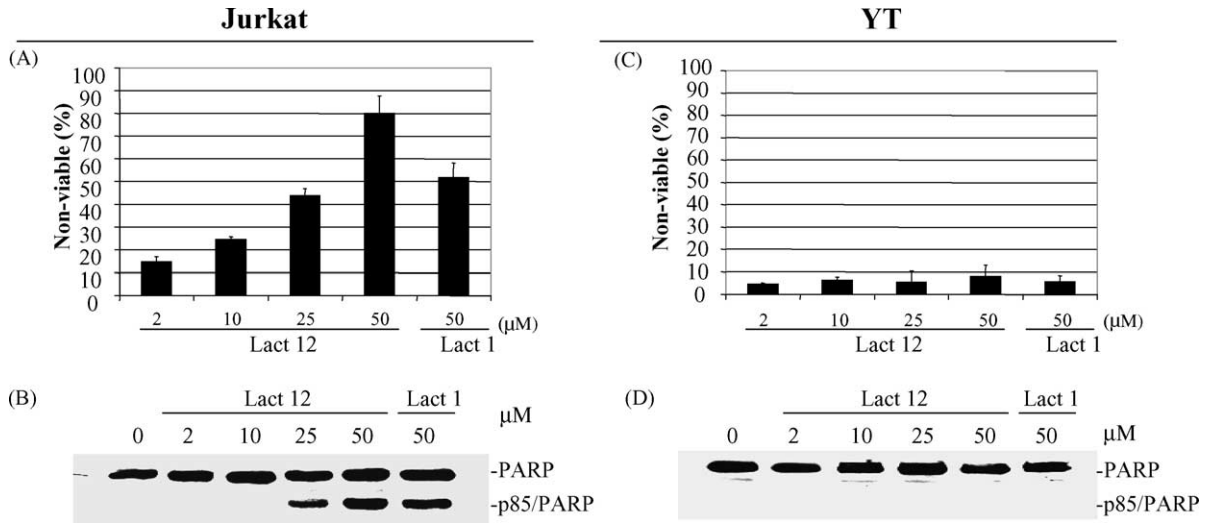

Fig. 3. Dose–response comparison between Jurkat T and YT cells treated with lactams **12** and **1** to induce cell apoptosis. Jurkat T (A and B) and YT cells (C and D) were treated with 2, 10, 25, and 50 μM of lactam **12** vs. 50 μM of lactam **1** for either 12 (B and D) or 24 hr (A and C), followed by trypan blue exclusion (A and C) or Western blot assay using anti-PARP antibody (B and D). Results are representative of three different experiments. Standard deviations are shown with error bars from a mean of at least three independent experiments (A and C).

assay using lysates prepared after 12-hr treatment (Fig. 3B). Cleavage of PARP occurred in lactam **12**-treated cells in a dose-dependent manner with the highest level of PARP cleavage observed at 50 μM (Fig. 3B). The levels of PARP cleavage induced by 50 μM of lactam **1** were equivalent to ~50% of that by 50 μM of lactam **12** (Fig. 3B).

In the same experiment, when immortalized, nontransformed NK cells were treated with lactam **12** (using lactam **1** as a control), neither cell death (Fig. 3C) nor PARP cleavage (Fig. 3D) were observed. Therefore, like lactam **1**, lactam **12** also induces apoptotic cell death preferentially in tumor *over* nontransformed cells.

To further compare the potency of lactams **1** and **12**, Jurkat T cells were treated with 25 μM of lactam **12** vs. 50 μM of lactam **1** for 3, 6, 12, and 24 hr, followed by determination of trypan blue incorporation and PARP cleavage. After 3 hr, lactam **12** at 25 μM caused 15% vs. 11% cell death with lactam **1** at 50 μM (Fig. 4A). Similarly, at 6 hr, 24% of trypan blue-positive cells were found after 25 μM lactam **12** treatment, while only 20% observed in 50 μM lactam **1**-treated cells (Fig. 4A). Only at later time points (12 and 24 hr), lactam **1** at 50 μM was slightly more potent than lactam **12** at 25 μM (Fig. 4A). Similar levels of cleaved PARP were observed in Jurkat T cells treated with either 25 μM of lactam **12** or 50 μM of lactam **1** at each time point (Fig. 4B). Therefore, lactam **12** is able to induce similar amounts of apoptosis in Jurkat T cells at a concentration half of that of lactam **1**.

Furthermore, we examined levels of sub-G_1 populations, as a measurement of cells with DNA fragmentation [18], in Jurkat T cells treated with lactam **12** or **1**. Treatment with 50 μM of lactam **12** increased the sub-G_1 populations by 34 and 57%, respectively, at 12 and 24 hr (Fig. 5A). In comparison, 50 μM of lactam **1** treatment for 12 and 24 hr induced sub-G_1 populations by 10 and 16%, respectively [17], confirming the greater potency of lactam **12**.

3.4. Lactam *12* is able to induce DNA damage in Jurkat T cells

We have previously shown that lactam **1** induces damage to DNA, leading to the inhibition of DNA replication and

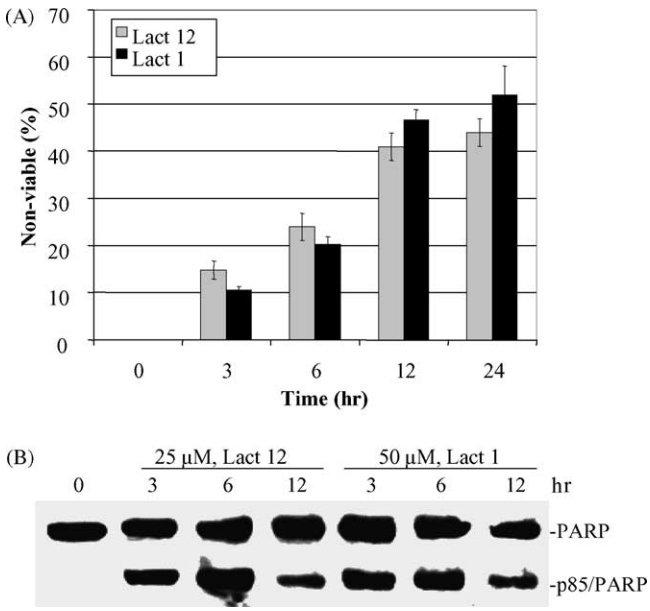

Fig. 4. A kinetic comparison between lactams **12** and **1** to induce apoptosis in Jurkat T cells. Jurkat T cells were treated with 25 μM of lactam **12** vs. 50 μM of lactam **1** for 3, 6, 12, and 24 hr, followed by trypan blue dye exclusion assay (A), and PARP cleavage in Western blot assay (B). Results are representative of three different experiments. Standard deviations are shown with error bars from a mean of at least three independent experiments.

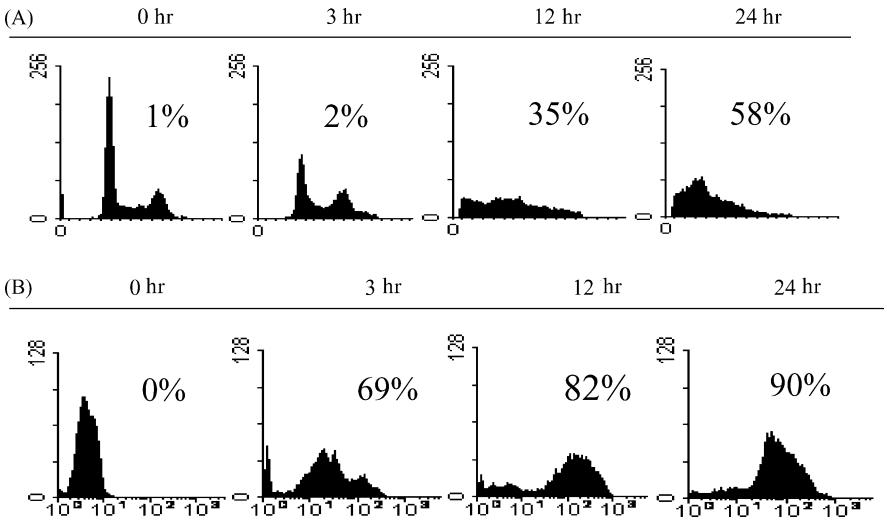

Fig. 5. Lactam **12** induces sub-G_1 cell population and TUNEL-positivity. Jurkat T cells (0 hr) were treated with 50 μM of lactam **12** for the indicated hours. (A) Measurement of sub-G_1 DNA content by flow cytometry analysis. The percentage of sub-G_1 cell population represents the cell populations with DNA fragmentation. (B) Measurement of DNA strand breaks by TUNEL assay. The numbers indicate the percentage of TUNEL-positive population. Results of representative of three independent experiments are shown.

subsequent induction of apoptosis [17]. To determine whether lactam **12** is also capable of damaging tumor cell DNA, Jurkat T cells were treated with 50 μM of lactam **12**, followed by performance of TUNEL assay, which detects DNA strand breaks [17]. A significant population (∼70%) of the cells exhibited DNA strand breaks after 3 hr of incubation with lactam **12** (Fig. 5B). A total of 82–90% of the cells were TUNEL-positive after 12–24-hr treatment with lactam **12** (Fig. 5B). In this experiment, 66% of TUNEL-positive cells were observed after treatment with 50 μM of lactam **1** for 24 hr (data not shown). Thus, the increased DNA-damaging capability of lactam **12** is most likely responsible for its enhanced cell death-inducing activity (Figs. 1–4).

*3.5. Lactams **1** and **12** induce apoptosis and inhibit colony formation in human prostate cancer cells*

So far, we demonstrated that lactam **12**, like lactam **1**, is able to induce DNA damage and subsequently induce apoptosis in human leukemia cells (Figs. 1–5) [17]. To determine if this lactam could also activate death program in solid tumor cells, human prostate cancer LNCaP cells were treated for 48 hr with lactam **12** at 2–25 μM or lactam **1** at 50 μM (as a control), followed by measurement of cell-free caspase-3/-7 activity. A dose-dependent increase in caspase-3/-7 induction was observed in LNCaP cells treated with lactam **12**: by 2-, 2.5-, and 4.2-fold, respectively, at 2, 10, and 25 μM (Fig. 6A). Treatment with 50 μM of lactam **1** also increased levels of caspase-3/-7 activity by 2.5-fold over the control (Fig. 6A). These data are consistent with the conclusion that lactam **12** has greater apoptosis-inducing potency than lactam **1**.

We then investigated the *in vivo* effects of these two lactams in a soft agar assay that measures the transforming activity of human tumor cells. LNCaP cells were plated in soft agar along with 50 μM of lactam **1**, 50 μM of lactam **12**, or solvent (DMSO), followed by a 21-day incubation to allow for colony formation. The solvent (DMSO)-treated plates allowed for the development of ∼500 colonies (Fig. 6B and C). Lactam **1** inhibited 91%, and lactam **12** completely blocked (∼100%), colony formation of LNCaP cells (Fig. 6B and C). Therefore, both lactams are able to inhibit the transformation capability of prostate cancer cells.

*3.6. Lactam **12** induces apoptosis in several solid tumor cell lines and SV-40-transformed, but not normal, human fibroblasts*

In a previous study, we showed that lactam **1** induced apoptosis in several solid tumor cell lines [17]. In this study we also investigated the effects of lactam **12** on several solid tumor cell lines including human breast (MCF-7), head-and-neck (PCI-13), and prostate (DU-145) cancer cells. Furthermore, we wanted to investigate whether lactam **12**-induced cell death was selective in transformed (VA-13) *over* the normal (WI-38) human fibroblasts. We treated these cell lines with 50 μM lactam **12** 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 change. We found that after a 48-hr treatment with lactam **12**, ∼60% of MCF-7 and PCI-13 cells and ∼50% of DU-145 and VA-13 cells became detached. However, no detachment was observed in WI-38 cells after treatment with lactam **12**. Little or no detachment was observed in all the cell lines treated with DMSO. All the detached tumor or transformed cells exhibited typical apoptotic nuclear condensation

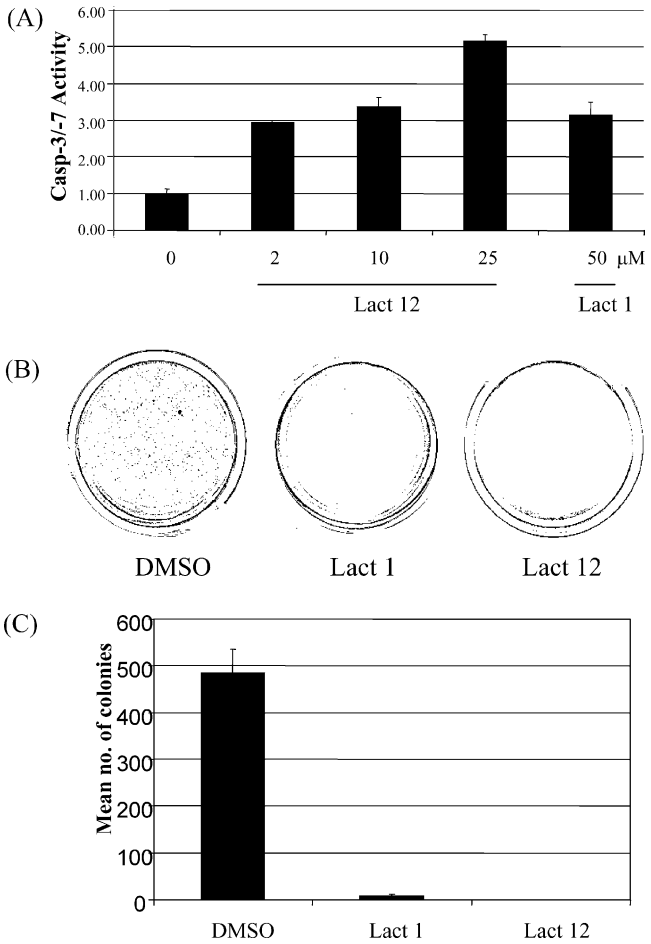

Fig. 6. Effects of β-lactams on caspase activation and colony formation. (A) Prostate cancer LNCaP cells were treated for 48 hr with 2, 10, and 25 μM of lactam **12** vs. 50 μM of lactam **1**. Cell-free caspase-3/-7 activity was then determined by incubating whole-cell extracts with caspase-3/-7 substrate and measuring free AMCs. (B and C) LNCaP cells were plated in soft agar with the solvent DMSO or 50 μM of the indicated β-lactams. Cells were then cultured for 21 days without addition of new drug. The plates were scanned and a representative well from each treatment was selected for presentation (B). Colonies were quantified with an automated counter and presented as mean values from triplicate independent experiments. Error bars denote standard deviations (C).

and fragmentation (Fig. 7A). In addition, apoptosis-specific nuclear condensation was also observed in the remaining attached solid tumor (MCF-7, PCI-13, and DU-145) and transformed (VA-13), but not the normal (WI-38), cells (Fig. 7A). These results strongly suggest that lactam **12** induces apoptosis that lead to detachment preferentially in the tumor and transformed cells.

To confirm lactam **12**-mediated apoptotic cell death, in the same experiment, aliquots of both detached and attached cells of each line were combined and used for whole-cell extract preparation. This was followed by measurement of cell-free caspase-3/-7 activity. Consistent with the apoptotic nuclear changes (Fig. 7A), treatment of MCF-7, PCI-13, DU-145, and VA-13 cells with lactam **12** also increased levels of caspase-3/-7 activity by 11.0-, 10.2-, 5.2-, and 5.3-fold, respectively, *over* the control DMSO-treated cells (Fig. 7B). In addition, accompanying the lack of the detachment in normal WI-38 cells treated with lactam **12** (Fig. 7A), there was little or no induction of caspase-3/-7 activity observed in these cells (Fig. 7B). Taken together, these data further support the conclusion that lactam **12** is able to induce apoptotic cell death preferentially in tumor and transformed *over* the normal cells.

4. Discussion

Developing novel anticancer drugs that induce apoptosis in tumor cells has long been a goal of cancer drug discovery research. Many of the drugs in current use focus on targeting dysregulated cell cycle and apoptosis programs in cancer cells [25]. We previously have shown that *N*-thiolated β-lactams cause DNA damage in tumor cells that leads to induction of apoptosis through p38 activation, cytochrome *c* release, and caspase activation [17]. Here we show that lactam **1** selectively induces apoptosis in human leukemic Jurkat T cells, but not nontransformed, immortalized human NK cells (Fig. 2). Furthermore, lactam **1** is capable of inducing Jurkat cell apoptosis at concentrations as low as 10 μM after 24-hr treatment (Fig. 2A).

Often, addition/substitution of groups on a molecule leads to development of more potent drugs. In order to determine whether structural changes to lactam **1** could produce a more potent tumor cell death inducer, analogs of lactam **1** were synthesized (Fig. 1A). Substitutions of the –Cl group with other halogens of higher atomic mass (–Br, –I) did increase the efficacy of the compound. In contrast, substitution with a lower atomic mass halogen (–F) or a hydrogen (H) atom had a concomitant decrease in cell death induction (Fig. 1). Lactam **12**, containing an $-NO_2$ substituent, proved to be a highly active compound and induced 93–100% of cell death at 50 μM vs. 52% of cell death by lactam **1** at the same concentration (Fig. 1B). Furthermore, lactam **12** was superior to lactam **1** at inducting apoptosis in human Jurkat T cells because lactam **12** can induce the same amount of PARP cleavage at a lower concentration than lactam **1** (Figs. 3 and 4). Additionally, lactam **12** at 25 μM was able to exert its cell death-inducing effect at as early as 3 hr (Fig. 4A and B). We also found that lactam **12** had greater potency than lactam **1** when used in human prostate cancer cells to activating caspase-3/-7 and inhibiting colony formation (Fig. 6). Similar to our previous results with lactam **1** [17], we found that lactam **12** induces apoptosis in several solid tumor cell lines (e.g. MCF-7, PCI-13, DU-145) in a caspase-dependent manner (Fig. 7). Due to lack of caspase-3 in MCF-7 cells, it was believed that lactam **12**-mediated MCF-7 cell death was associated with caspase-7 activity (Fig. 7). Additionally, like lactam **1**, lactam **12** was also able to selectively induce apoptosis in human leukemic Jurkat T cells *over* nontransformed,

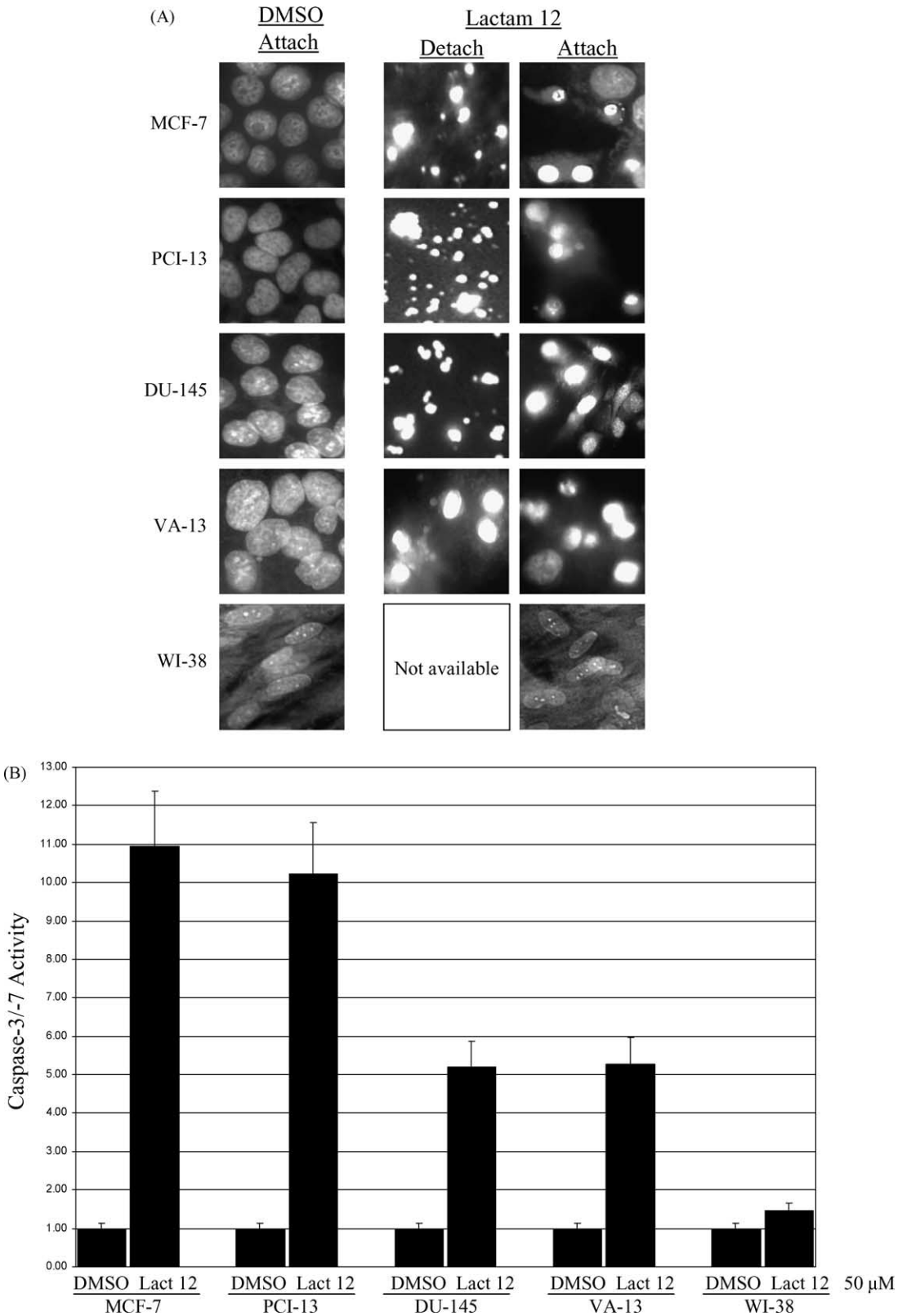

Fig. 7. Lactam **12** induces apoptosis and caspase activation in different solid tumor cell lines and SV40-transformed but not normal cells. (A) Nuclear staining assay. MCF-7, PCI-13, DU-145, VA-13, and WI-38 cells were treated with 50 μM lactam **12** or DMSO for 48 hr, followed by collection of both detached and attached cell populations. After lactam **12** treatment, ~60% of MCF-7 and PCI-13 cells and ~50% of DU-145 and VA-13 cells became detached, whereas <5% were detached from each of these cell lines after DMSO treatment. No detachment was found in WI-38 cells after each treatment. Both detached and attached cell populations were stained with the nuclear staining dye Hoechst 33342. Each sample was then analyzed by fluorescence microscopy for nuclear morphology. (B) Cell-free caspase-3/-7 activity assay. Aliquots of the above detached and attached cells of each line were combined for whole-cell extraction. Cell-free caspase-3/-7 activity was then determined by incubating each whole-cell extract with caspase-3/-7 substrate and measuring free AMCs. Error bars denote standard deviations. Similar results were obtained in three independent experiments.

immortalized human NK cells (Fig. 3). Also, lactam **12** was able to selectively induce apoptotic cell death in simian virus 40-transformed, but not the parental normal, human fibroblasts (Fig. 7). The molecular mechanism for the enhanced activity in lactam **12** remains unknown. One interpretation is that the presence of $-NO_2$ group in this drug increases its binding to the cellular target(s). Alternatively, this drug might have increased uptake rates by the cells.

The mechanism of action of many chemotherapeutic drugs is through DNA damage and then subsequent apoptosis induction in tumor cells [26]. As mentioned above, we have recently shown that lactam **1** is capable of inducing apoptosis after DNA damage [17]. In the present study, we show, by TUNEL assay, that lactam **12** also causes DNA damage in ~70% of cells just after 3-hr treatment (Fig. 5B). At this time, there was only 2% cell death (Fig. 5A), suggesting that the DNA damage occurs much earlier than apoptotic cell death. However, apoptotic cells increased at later time points with increased TUNEL-positive cells (Fig. 5A and B). This result is consistent with our previous study [17] and several other studies that have shown that several traditional chemotherapeutic drugs or DNA-damaging agents cause DNA strand breaks that trigger apoptotic cell death [27,28].

Malignant transformation of a cell can lead to tumor formation and metastasis. The desired effect of any anticancer drug is to inhibit tumor growth and formation *in situ*. Soft agar colony forming assay is an assay that has been developed to mimic tumor cellular growth in tissue. We hypothesized that the *N*-thiolated β-lactams that induce cell death should be able to inhibit colony formation in soft agar assay. Indeed, when LNCaP prostate cancer cells were cultured in the presence of lactam **1** or **12**, 91 and 100% inhibition of colony formation was observed, respectively, as compared to the solvent control (Fig. 6).

Based on our previous [17] and current studies, we propose that these *N*-thiolated β-lactams act by inducing DNA damage that leads to apoptosis preferentially in cancer and transformed *over* normal/nontransformed cells. Although it appears that the *N*-methylthio moiety is necessary for the cell death-inducing activity [17], addition of a larger group in the *ortho* position on the phenyl ring can also increase the effectiveness of the compound (Fig. 1). Our results strongly suggest the potential for developing this class of β-lactams into novel anticancer agents. Immediate future studies focusing on determining the molecular targets and chemical action of the *N*-thiolated β-lactams would help rational development of these compounds.

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