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STRUCTURE-ACTIVITY RELATIONSHIPS OF *N*-METHYLTHIOLATED BETA-LACTAM ANTIBIOTICS WITH C_3 SUBSTITUTIONS AND THEIR SELECTIVE INDUCTION OF APOPTOSIS IN HUMAN CANCER CELLS

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1. ABSTRACT

The development of novel anti-cancer drugs that induce apoptosis has long been a focus of drug discovery. Beta-lactam antibiotics have been used for over 60 years to fight bacterial infectious diseases with little or no side effects observed. Recently a new class of *N*-methylthiolated beta-lactams has been discovered that have potent activity against methicillin resistant *Staphylococcus aureas*. Most recently, we determined the potential effects of these *N*-thiolated beta-lactams on tumorigenic cell growth and found that they are apoptosis-inducers in human cancer cell lines. In the current study, we further determined the effects of the substitution of the *O*-methyl moiety on C_3 and stereochemistry of the beta-lactams on the anti-proliferative and apoptosis-inducing abilities. We have found that lactam 18, in which C_3 is substituted with an acrylate ester group, is a very effective proliferation inhibitor against human premalignant and malignant breast, leukemic, and simian virus 40-transformed fibroblast cells. Generally speaking, increasing the size of the moiety on C_3 decreases its anti-proliferation potency, possibly indicating steric hindrance with the cellular target or decreased permeability through the cell membrane. We also found that the stereochemistry of the beta-lactams plays an important role in their potency. The 3S,4R isomers are more effective than their enantiomers (3R,4S), suggesting that 3S,4R configuration is more favorable for target interaction.

2. INTRODUCTION

Selectively targeting tumorigenic cells *versus* normal cells is a primary goal in anti-cancer drug discovery. Small molecules with apoptosis-inducing ability have great potential to be developed into novel chemotherapeutic drugs because of the ease of synthesis and structural manipulation (1-3). Initiation, commitment, and execution are the three fundamental steps of apoptosis (4). Several apoptotic stimuli, such as irreparable DNA damage, signal to activate the initiator caspases (*e.g.* caspases-8/10), which in turn activate downstream effector

caspases (*e.g.* caspases-3/7). The effector caspases can also be activated through the release of mitochondrial proteins, such as cytochrome *c* (5). It is generally believed that proteolytic cleavage of a variety of intracellular substrates by effector caspases leads to apoptosis (6-9).

One particularly important class of small molecule drugs, the beta-lactam antibiotics, have played an essential role in treating bacterial infections without causing toxic side effects in the host for the past 60 years. Sir Alexander Fleming first coined the name "penicillin" in 1928 after his discovery that molds from the *Penicillium* genus secrete powerful antimicrobial compounds, called beta-lactams (10). X-ray crystallography revealed that penicillin is a thiazolidine ring fused to a four membered beta-lactam ring (11). The beta-lactams are powerful and potent inhibitors of bacterial growth and many different moieties of bicyclic beta-lactams have been isolated or synthesized since the discovery of penicillin (12). There are several classes of bicyclic beta-lactams that possess antibacterial properties, including the penams, penems, carbapenems, cephalosporins, and clavulanic acids (10).

A novel class of beta-lactams was discovered by the Squibbs and Takeda laboratories in 1981, which have an *N*-sulfonic acid group attached directly to the nitrogen in the lactam ring (13 , 14). The term "monobactam" was coined for these lactams, which have a flexible monocyclic ring and lack the carboxylic acid moiety, yet still retain a high bactericidal potency. Recently, a structurally related family of *N*-thiolated compounds, termed *N*-methylthio beta-lactams, was found to inhibit growth of *Staphylococcal* and methicillin-resistant *S. aureus* (MRSA) (15-17). Additionally, we have shown that these *N*-methylthio beta-lactams possess potent anti-proliferative properties, and are capable of inducing DNA strand breakage, inhibiting DNA replication, and inducing apoptosis in a time- and concentration-dependent manner when tested in several human cancer, but not normal cell lines (18 , 19).

In this study, we screened several additional *N*-thiolated beta-lactams with substitutions made to the *O*-methyl moiety of carbon 3 (C_3) for their structure-activity relationships and found that increasing the size of the C_3 substitution results in decreased anti-proliferative activity in human breast cancer cells. Additionally, increasing the size of the C_3 substituent may interfere with cellular uptake. We identified one particularly active lactam (lactam **18**), which possesses an acrylate ester moiety off of C_3, for further study. Lactam **18** induces caspase-3 activation and apoptosis, associated with increased Hsp70 protein expression and p38 phosphorylation. We have also found that the stereochemistry plays an important role in the activities of *N*-thiolated beta-lactam antibiotics, including anti-proliferation, S/G_2/M cell cycle arrest, and apoptosis induction. The 3S,4R-configured [(+)] isomers of lactam **18** and lactam **19** are more potent than their 3R,4S-configured isomers or the racemic mixtures. Furthermore, these (+)-lactams are more efficacious than racemic lactam **1**, which was identified from our previous studies (18). These effects of beta-lactams were found mainly in cultured human cancer and transformed cells, but not in normal/non-transformed cells. These data indicate that further study of *N*-thiolated beta-lactams in the treatment of cancers is warranted.

MATERIALS AND METHODS

3.1. Reagents

Fetal Bovine Serum was purchased from Tissue Culture Biologicals (Tulare, CA). Mixture of penicillin-streptomycin-L-glutamine, RPMI, Dulbecco's modified Eagle's medium (DMEM), DMEM/F12 (1:1) medium, horse serum, MEM non-essential amino acids solution, MEM sodium pyruvate solution, penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA). (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO), epidermal growth factor (EGF), sodium bicarbonate, hydrocortisone, cholera enterotoxin, bovine insulin, propidium iodide and trypan blue were purchased from Sigma-Aldrich (St. Louis, MO). Polyclonal antibodies to actin, monoclonal antibodies to HSP70 and p-p38, and anti-goat and anti-mouse IgG-horseradish peroxidase were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). CaspACE FITC-VAD-FMK marker was purchased from Promega (Madison, WI). Fluorogenic peptide substrate Ac-DEVD-AMC (for caspase-3/-7 activities) was obtained from Calbiochem (San Diego, CA).

3.2. Synthesis of beta-lactams

The beta-lactam analogs (Figure 1) were prepared as racemates and enantiomers (with *cis* stereochemistry) using a procedure described previously (15 , 16).

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

Human leukemic Jurkat T cells and natural killer YT cells were cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Further supplementation with 1 mM MEM sodium pyruvate solution, 0.1 mM MEM non-essential amino acids solution was added to YT cells. Human breast cancer MCF-7 cells, normal (WI-38) and SV-40 transformed (VA-13) human fibroblasts cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, penicillin, and streptomycin. Premalignant MCF10AT1Kcl.2 transformed human breast cells (20) were cultured in DMEM/F12 (1:1) supplemented with 10 μg/ml bovine insulin, 100ng/ml cholera enterotoxin, 20 ng/ml epidermal growth factor, 500 ng/ml hydrocortisone, 29 mM sodium bicarbonate, 5% horse serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. All cell lines were maintained at 37°C in a humidified incubator with an atmosphere of 5% CO_2. A whole-cell extract was prepared and Western blotting was performed as described previously [18].

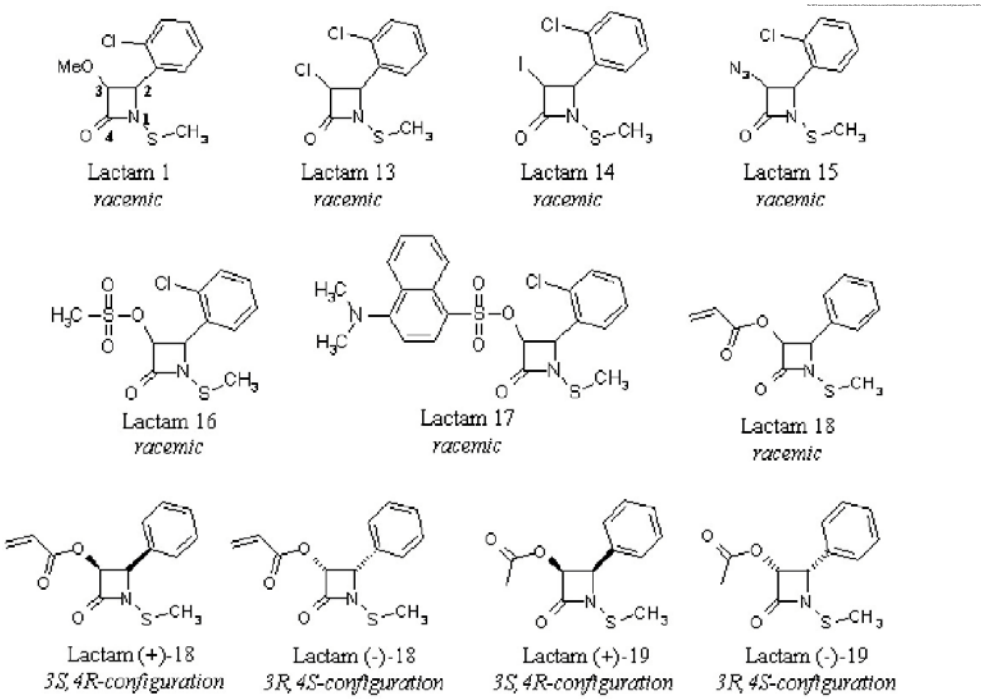

Figure 1. Chemical structures of *N*-methylthiolated beta-lactams.

3.4. Cellular proliferation assay

The MTT assay was used to determine the effects of beta-lactams on overall proliferation of tumor cells. Cells were plated in a 96-well plate and grown to 70-80% confluency, followed by addition of each compound at an indicated concentration for 24 h. MTT (1 mg/ml) in PBS was then added to wells and incubated at 37ºC for 4 hours to allow for complete cleavage of the tetrazolium salt by metabolically active cells. Next, MTT was removed and 100 □l of DMSO was added, followed by colorimetric analysis using a multilabel plate reader at 560 nm (Victor3; Perkin Elmer). Absorbance values plotted are the mean from triplicate experiments.

3.5. 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.

3.6. Cell cycle assay

Cell cycle analysis based on DNA content was performed as follows. Cells were harvested, counted, and washed twice with PBS. Cells (5×10^6) were then suspended in 0.5 ml of PBS, pipetted, and fixed in 5 ml of 70% ethanol for at least 2 h at -20°C. Cells were centrifuged, resuspended in 1 ml of propidium iodide staining solution (50 µg propidium iodide, 1 mg RNase A, and 1 mg of glucose per ml of PBS) and incubated at room temperature for 30 min before flow cytometry analysis. 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.

3.7. Caspase-3 activity assay

To measure cell-free caspase-3 activity, whole cell extracts (30 µg) from untreated or treated cells were incubated with 20 µM of the fluorogenic substrate caspase-3/-7 (Ac-DEVD-AMC) for 30 min at 37°C in 100 µl of assay buffer (50 mM Tris, pH 8.0). Measurement of the hydrolyzed AMC groups was performed on a Victor^3 Multilabel plate reader^{TM} (Perkin Elmer) as described previously [18].

3.8. Immunostaining of apoptotic cells

Immunostaining of apoptotic cells was performed by addition of the FITC-VAD-FMK marker and visualized on an Axiovert 25 microscope (Zeiss; Thornwood, NY). Briefly, cells were grown to ~80% confluency in 60 mm dishes, and then treated under conditions described in the figure legends. Detection of caspase activity was determined according to the manufacturer's protocol with a few modifications. Briefly, total cell population was collected and incubated with a 10 µM FITC-VAD-FMK for 20 min in the dark. Cells were then centrifuged at 300 xg for 3 minutes, washed 3X in PBS, and then resuspended in 50 µl PBS. Cell suspension was then transferred to glass slides in the presence of Vector Shield mounting medium with DAPI. Images were captured using AxioVision 4.1 and adjusted using Adobe Photoshop 6.0 software. Apoptotic cells were quantified by counting the number of

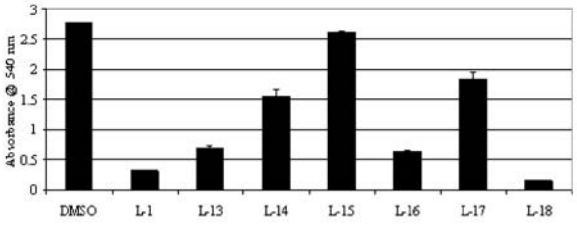

Figure 2. Structure-activity relationship (SAR) analysis of *N*-thiolated beta-lactams. MCF-7 cells were plated in a 96-well plate and grown to 70-80% confluency followed by addition of 50 μM beta-lactam for 24 h. Cells were then incubated with 1 mg/ml MTT for 3 h and proliferation rates were determined using a muli-label plate reader (Victor^3, Perkin Elmer; ±SD).

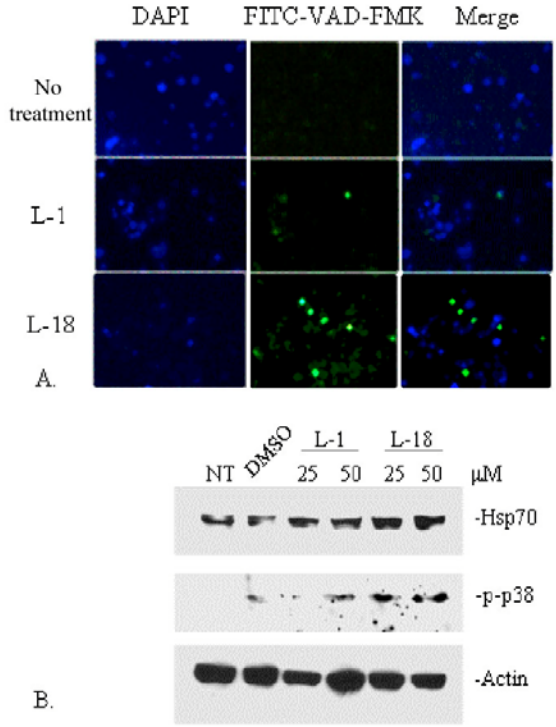

Figure 3. Lactam **18** induces caspase activity associated with Hsp70 expression and p38 phosphorylation. **A**. Jurkat T cells were treated with 20 μM lactam **1** or lactam **18** for 24 h. Following the treatment, the cells were then incubated with a FITC-conjugated marker that binds to activated caspases. Cell suspension was then transferred to glass slides in the presence of Vector Shield mounting medium with DAPI. Images were captured using AxioVision 4.1 and adjusted using Adobe Photoshop 6.0 software. **B**, Jurkat cells treated with 25 or 50 μM of lactam **1** or lactam **18** for 16 h, followed by Western blot analysis using specific antibodies to HSP70, p-p38, and Actin. Data shown are representative from three independent experiments.

apoptotic cells *over* the total number of cells in the same field.

3.9. Nuclear staining

After each drug treatment, both detached and attached populations of VA-13 and WI-38 lines were stained with Hoechst 33342 to assess apoptosis. Briefly, cells were washed 2X in PBS, fixed for 1 h with 70% ethanol at 4ºC, washed 3X in PBS, and stained with 50 μM Hoechst for 30 min in the dark at room temperature. Detached cells were plated on a slide and attached cells were visualized on the culture plate with a fluorescent microscope at 10X or 40X resolution (Zeiss, Thornwood, NY). Images were obtained using an AxioVision 4.1 and adjusted using Adobe Photoshop 6.0.

4. RESULTS

4.1. Structure-activity relationship analysis of *N*-thiolated beta-lactams

We have previously shown that lactam **1**, which contains an *O*-methyl moiety at carbon-3 (C_3) of the beta-lactam ring, induces apoptosis in a relatively selective manner in tumor and transformed cells, but not normal or non-transformed cell lines (18). More than 35 *N*-thiolated beta-lactam analogs were then screened using an MTT assay in breast cancer MCF-7 cells to assess their anti-proliferative potency compared to lactam 1. Several of these analogs with substitutions to the *O*-methyl group at C_3 (Figure 1) were then selected for further structure-activity relationship (SAR) studies (Figure 2). MCF-7 cells were treated with 50 μM of selected lactams for 24 h, followed by MTT assay. Lactam 18, which possesses an ester moiety at C_3, is twice as potent as lactam 1 (Figure 2). Additionally, it was found that as the C_3 group increased in size, the effectiveness to inhibit proliferation decreased. For instance, increasing the size of the halogen, from –Cl to –I (lactam 13 *versus* lactam 14), lead to a 2-fold decrease in potency (Figure 2). A similar observation was made in comparing the bioactivities of C_3-sulfonated lactams 16 and 17. Mesyl lactam 16 inhibited 57% of MCF-7 cell growth, while dansyl lactam 17 has much less effect on inhibiting proliferation (Figure 2). A possible explanation may be that lactam 17 with the large dansyl group is incapable of crossing the cell membrane. Although lactam 15 with an N_3 group at C_3 is less potent than lactam 14 with I at C_3, as predicted, it is yet unclear why lactam 15 is less potent than mesyl lactam 16 (Figure 2). The order of potency is determined as follows: Lactam 18 $>$ 1 $>$ 16 $\geq$ 13 $>$ 14 $>$ 17 $>$ 15.

4.2. Lactam 18 is more potent than lactam 1 at inducing apoptosis associated with Hsp70 expression and p38 phosphorylation

We decided to focus on lactam **18** due to its increased anti-proliferative potency *over* lactam **1** (Figure 2). To determine whether lactam 18 is capable of inducing apoptosis, we treated leukemia Jurkat T cells with 20 μM lactam 18 for 24 h, using lactam 1 as a control. A fluoroscent marker specific for activated caspases was then added to the cells and then visualized by fluorescence microscopy (Figure 3A). Lactam 18 displayed greater apoptosis-inducing activity than lactam 1 (Figure 3A).

Microarray analysis showed up-regulation of many genes by lactam 1 treatment, including *HSP70*

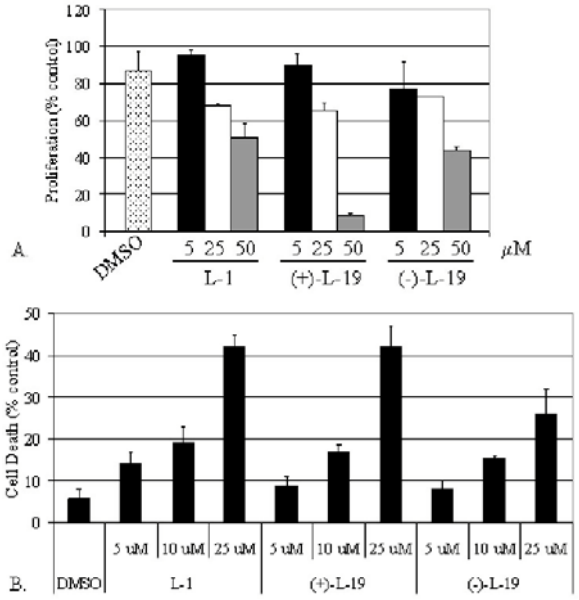

Figure 4. (+)-Lactam **19** effects proliferation and cell death in a dose-dependent manner. **A**, MCF10AT1Kcl.2 cells were plated in a 96-well plate and grown to 70-80% confluency followed by addition of 50 μM of indicated beta-lactams for 24 h. Cells were then incubated with 1 mg/ml MTT for 4 h and proliferation rates were determined using a muli-label plate reader (Victor^3, Perkin Elmer; ±SD). **B**, Jurkats T cells treated with lactam **1**, (+)-lactam **19,** (-)-lactam **19** at indicated doses and assayed for cell death by trypan blue incorporation (±SD).

(manuscript in preparation). To confirm this finding, Jurkat T cells were treated with lactam 1 and lactam 18 for 16 h at either 25 or 50 μM. It was found that Jurkat T cells had increased Hsp70 protein expression after treatment of both lactams; lactam 18 induced a greater amount of Hsp70 expression at 25 μM than lactam 1 at the same concentration (Figure 3B). This is consistent with the idea that the beta-lactams induce a stress response in the cell, most likely due to their disruption of DNA synthesis (18).

We have previously reported that p38 MAP kinase activation is associated with β-lactam-induced apoptosis (18). Activation of p38 MAP kinase can trigger apoptosis following multiple stimuli, such as DNA damage (21 , 22). Western blot for phosphorylated (activated) pp38 reveals that treatment with 25 μM lactam 18 leads to an increase in pp38 levels (9.0-fold), compared to that of 25 μM lactam 1 (2.6-fold) (Figure 3B). Actin was used as a loading control.

4.3. (+)-Lactam 19 inhibits cellular proliferation more effectively than (-)-lactam 19

To determine if the stereochemistry has any bearing on the potency of beta-lactams, lactams 18 and 19 were synthesized in enantiomerically pure forms (Figure 1). Premalignant MCF-10AT1Kcl.2 breast cancer cells were treated with (+)-lactam 19 (3S,4R-configuration), (-)-lactam 19 (3R,4S-configuration), or racemic lactam 1 (as a control) for 24 h at indicated doses (Figure 4A). All of these lactams inhibited proliferation in a dose-dependent manner, with (+)-lactam 19 at 50 μM inhibiting 85% cell growth, (-)-lactam 19 inhibiting 56% and lactam 1 inhibiting 49% at the same concentration (Figure 4A). These results are similar to that from another experiment using malignant MCF-7 breast cancer cells (data not shown). Trypan blue incorporation also shows increased tumor cell killing with (+)-lactam 19 to that of (-)-lactam 19, 42% versus 26%, respectively, at 25 μM (Figure 4B).

4.4. beta-Lactams 18 and 19 induce tumor cell-selective apoptosis

We have previously shown that lactam 1 preferentially induces apoptosis in human cancer cells over normal, non-transformed cells lines (19). To determine if lactam 18 possessed a similar tumor cell-specific activity, human leukemic Jurkat T cells and immortalized, non-transformed natural killer cells (YT) were treated with lactam 1 and lactam 18, and the effects were determined. After 16 h treatment, it was found that lactam 18-treated Jurkat cells had a 19-fold increase in caspase-3 activity at 25 μM, compared to 9-fold induced by lactam 1 at the same concentration (Figure 5A). Both lactam 18 and lactam 1 had little or no apoptosis-inducing effects on the immortalized, non-transformed YT cells (Figure 5A).

To assess whether the isomers of lactam 19 (Figure 1) also have explicit activity against cancer cells, we treated Jurkat T cells and YT cells with (+)-lactam 19, (-)-lactam 19 and racemic lactam 1 (as a control), and determined the effects on apoptotic cell death. We found that only the Jurkat, but not YT, cells exhibited high levels of caspase-3 activity when treated with these beta-lactams (Figure 5B). Additionally, (+)-lactam 19 at 25 μM induced a 7.4-fold increase in caspase-3 activity compared to 5.5-fold increase by (-)-lactam 19 and 3.1-fold increase by lactam 1 at 25 μM (Figure 5B). Therefore, the 3S,4R-configured isomer, (+)-lactam 19, is more potent than (-)-Lactam 19 and racemic lactam 1.

To further confirm the effect of stereoselectivity on the apoptosis-inducing effects of beta-lactams, we synthesized an isomeric pair of lactam 18, 3S,4R-isomer [(+)-lactam 18] and 3R,4S-isomer [(-)-lactam 18] (Figure 1). Jurkat and YT cells were then treated with 50 μM (+)-lactam 18, (-)-lactam 18, racemic lactam 18 or lactam 1 for 24 h, followed by trypan blue dye exclusion assay (Figure 6A). (+)-lactam 18 induced much higher amount of cell death than its isomer (-)-lactam 18, 98% *vs.* 58%, respectively (Figure 6A). Interestingly, the racemic lactam 18 was almost equally potent to that of (+)-lactam 18 (Figure 6A). All lactam 18 compounds initiated more cell death than lactam 1 (Figure 6A). Again, it was found that normal, non-transformed YT cells did not undergo cell death after treatment with any of these beta-lactams (Figure 6A).

Another experiment using SV-40 transformed (VA-13) and normal (WI-38) human fibroblasts demonstrates again that (+)-lactam 18 is the more active isomer. A nuclear stain of VA-13 and WI-38 cell lines treated with 50 μM of each beta-lactam for 24 h reveals

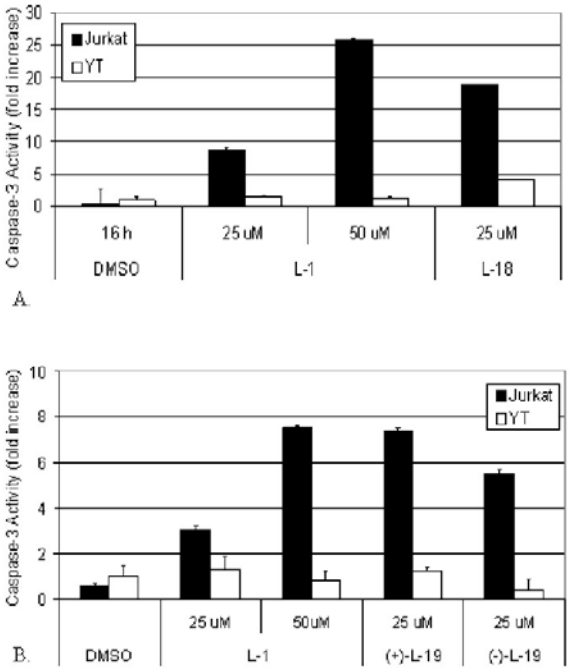

Figure 5. beta-Lactams induce apoptosis in a tumor cell-specific manner. **A**, Jurkat T and YT cells were treated with lactam **1** and lactam **18** at indicated concentration for 16 h, followed by measurement of cell-free caspase-3 activity by incubating whole cell extracts with caspase-3 substrate and measuring free AMCs. **B**, (+)-lactam **19** is 2-fold more potent that lactam **1** at inducing apoptosis in a tumor cell specific manner. Jurkat T and YT cells were treated for 24 h with 25 and 50 μM of lactam **1** *versus* 25 μM of (+)-lactam **19** and (-)-lactam **19**. Cell-free caspase-3 activity was then determined by incubating whole cell extracts with caspase-3 substrate and measuring free AMCs.

that there is a high degree of detachment and DNA condensation, characteristics indicative of apoptosis, in cells treated with (+)-lactam 18, racemic lactam 18 and lactam 1 (Figure 6B). (-)-lactam 18, on the other hand, showed decreased activity when compared with (+)-lactam 18 and racemic lactam 18. There was a very minor amount of cellular detachment observed in the normal WI-38 fibroblasts treated with (+)-lactam 18 (data not shown), supporting that these beta-lactams selectively kill transformed VA-13 cells (Figure 6B).

4.5. (+)-Lactam 18 induces S/G2/M cell cycle arrest

We have previously shown that beta-lactams decrease G_1 population, associated with DNA damage (18). To further investigate the cause of apoptosis after *N*-methlythio beta-lactam treatment, analysis of cell cycle changes were performed on an exponentially growing cell population (Table 1). As a control, lactam 1 was found to decrease G_1 phase DNA content by 6% after 6 h incubation (Table 1). Racemic lactam 18 had a very similar effect on cell cycle as racemic lactam 1 (Table 1). However, when cells were treated with (+)-lactam 18, there was a 15% decrease in G_1, demonstration that the 3S,4R configuration has increased growth-inhibitory activity (Table 1).

5. DISCUSSION

Currently, many anticancer therapies, from radiation treatment to chemotherapeutic agents, are very toxic. Therefore, drug discovery for anticancer therapy is as concerned with selectivity of normal *versus* cancer tissues, as it is the potency of the therapy itself. Antibiotic therapies have typically used the unique molecular targets of microbes in order to avoid toxicity to the patient during treatment. Recently we have shown that some of these compounds possess anti-proliferation activity in human tumor cells (18). Thus, these compounds that are already known to be essentially non-toxic to humans may be anticancer agents as well. Of particular interest are the *N*-thiolated beta-lactams, which we have previously found to be potent against MRSA (17). Additionally, we found these compounds act as potential anticancer agents through S-phase arrest, DNA damage, and apoptosis induction (18). These compounds are also able to selectively induce apoptosis in cancerous *over* normal cells (19).

This novel class of *N*-thiolated beta-lactams possesses potent anti-cancer activity, which is directly related to the nature of the substituents on each of the four ring sites. We have previously reported on the effects of additions/substitutions to the *N*-thio group and aryl ring (18 , 19). The work reported here is a further characterization of the SAR between the various substitution groups on beta-lactam ring. At the core of the beta-lactam molecule is a four-membered ring that is substituted at each position (Figure 1). Each of the positions, we have previously published, plays a role in the potency of the compound. Position 1 is the N-methythio position and changes at this position that either eliminate the methylthio moiety or lengthen the chain result in decreased potency (18). Position 2 is substituted with a benzene ring and changes here also effect potency (19). Physical position on the ring with regards to ortho-, para-, or meta substitution as well as the nature of the substituent affected the potency dramatically [for details see (19)]. Position 4, which is substituted with a double-bonded oxygen, is the "backbone" of the beta-lactam and therefore cannot be changed without losing the general beta-lactam framework (10 , 23). The work presented here examines the SAR at position 3 and completes the survey of each position of the four-membered ring.

We determined that the size and polarity of the group at C_3 is important for the lactam's activity. As these C_3 substituents increase in size or in polarity, the efficacy of the compound seems to drop. For example, replacing the chloro (Cl) moiety of lactam 13 for azido (N_3), lactam 15, decreases the anti-proliferative activity from 75 to 5% respectively (Figure 2). However, a simple single atom change in the same period (Cl to I; lactam 13 *vs.* lactam 14) seems to result in only a partial increase in potency. Similarly, the potencies of C_3-sulfonated compounds 16 and 17 can be directly attributed to their C_3 substitutions: lactam 17 with its large, polar dansyl moiety has significantly diminished activity compared to its smaller, less polar mesyl analog, lactam 16 (Figure 2). Likewise, lactam 16 seems to be similar in or slightly less potent than

Table 1. Cell cycle analysis of asynchronous Jurkat T cells treated with beta-lactams at 50 μM for 6 h.[1]

	No treatment	Lactam **1**	Lactam **18**	(+)-Lactam **18**
% G_0/G^1	42	36	36	27
% S	42	38	41	46
% G2/M	16	26	23	27
% G0/G1 Δ^2	--	-6	-6	-15

[1] The cell cycle distribution was measured as the percentage of cells that contain G_1, S, G_2 and M DNA ($G_1/S/G_2/M$ = 100%). [2] The percent change from control cells (no treat) is shown as % Δ.

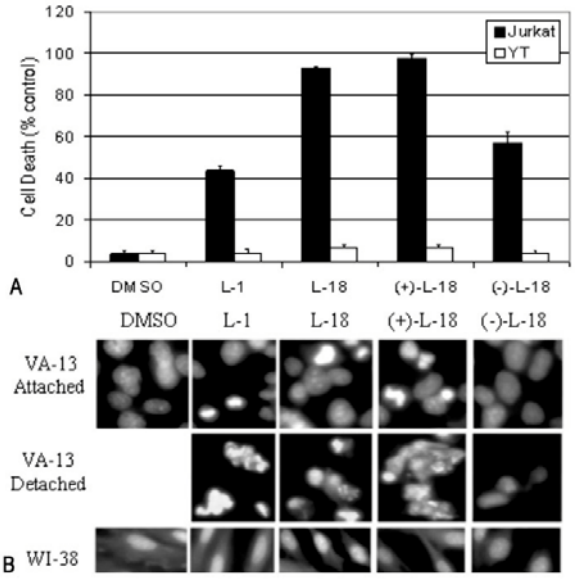

Figure 6. (+)-Lactam **18** induce apoptosis selectively in tumorigenic cells. **A**, Leukemic Jurkat T and non-transformed YT cells were treated with lactam **1** or isomers of lactam **18** at 50 μM for 24 h. Cell death is given as a percent of dead cells over total cell population (±SD). **B**, Both detached and attached VA-13 and WI-38 fibroblast cell populations were collected and stained with the nuclear staining dye Hoechst 33342. Each sample was then analyzed by fluorescence microscopy for nuclear morphology.

lactam 14, indicating that the size of the substituent may be slightly more significant than the overall charge (Figure 2). Lactam 18 displays very potent activity with its acrylate ester off C_3. This may indicate that these substitutions may affect the capability of these compounds to cross the cell membrane.

Of primary importance in anticancer drug research is that the compound being investigated demonstrate selectivity between normal cells and tumor cells. Cytotoxic agents are less desirable than those compounds that can differentially activate apoptosis in cancer cells *vs.* tumor cells. Previously we have reported that lactam 1-induced apoptosis is caspase-dependent and associated with cytochrome c release (18). Here we show that lactam 18-induced apoptosis is also caspase-dependent (Figure 5A). However, the efficacy to induce apoptosis by lactam 18 is much improved over lactam 1 (Figure 3) and that the apoptosis induced by lactam 18 is tumor cell-specific (Figure 5A). beta-Lactams cause DNA strand breakage and subsequent cell cycle arrest (18). Our microarray studies show a 3.5-fold increase in *HSP70* expression in Jurkat T cells treated with lactam 1 (data not shown). Increased expression of Hsp70 protein (Figure 3B) indicates that treatment with lactam 18 induces a stress response in leukemic Jurkat T cells. Another important molecular event in lactam-induced apoptosis is the increase in p38 phosphorylation. Abrogation of pp38 activity with a specific inhibitor (PD169316) leads to tumor cell survival (18). Not only is lactam 18 capable of inducing p-38 activation, it is capable of inducing a greater amount of pp38 levels at 25 μM compared to 50 μM of lactam 1 (Figure 3B).

Stereochemistry can play an important role in the efficacy of a particular compound. Often only one of the isomers displays a significant selectivity for the molecular target while the other can cause adverse side effects (24-26). Here we find that two 3S,4R-configured beta-lactam compounds, (+)-lactam 18 and (+)-lactam 19, do have a higher potency than their 3R,4S enantiomers or a racemic mixture. Specifically, (+)-lactam 19 has greater anti-proliferation and cell death-inducing activities than both (-)-lactam 19 and the racemic lactam 1 (Figure 4). (+)-Lactam 19 triggers an equivalent amount of capase-3 activation at half the concentration of lactam 1 and this activity again is tumor cell-selective (Figure 5). Additionally, another isomer, (+)-lactam 18, displays a similar potency to (+)-lactam 19 while still retaining the tumor cell-selectivity (Figures 5B and 6).

A vast amount of anti-cancer research is ongoing to develop apoptosis-inducing drugs. While the molecular targets and chemical actions of *N*-thiolated beta-lactams are not fully characterized, we believe that the compounds possess great potential for chemotherapeutic drug development. These antibiotics compounds are predicted to have little to no effect on normal cells, supported by our results. Thus, the anti-tumor potential and expected lack of toxicity of these beta-lactams makes them excellent candidates for anticancer drug development. Our ongoing studies focus on identifying the molecular interactions of beta-lactams in human cancer cells and their anti-tumor activities *in vivo*.

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