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Anti-tumor activity of *N*-thiolated β -lactam antibiotics

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Abstract

An ongoing strategy for cancer treatment is selective induction of apoptosis in cancer over normal cells. *N*-thiolated β -lactams were found to induce DNA damage, growth arrest and apoptosis in cultured human cancer cells. However, whether these compounds have a similar effect *in vivo* has not been studied. We report here that treatment with the β -lactam L-1 caused a significant inhibition of tumor growth in a breast cancer xenograft mouse model, associated with induction of DNA damage and apoptosis *in vivo*. These results suggest that the synthetic antibiotic *N*-thiolated β -lactams hold great potential to be developed as novel anti-cancer drugs.

Keywords

-Lactam; DNA damage; Apoptosis; Anti-tumor activity; Breast cancer

1. Introduction

Apoptosis, or programmed cell death, is an evolutionarily conserved cellular suicide program essential for the development of multi-cellular organisms [1]. By removing unwanted cells, apoptosis plays a vital role in development, tissue homeostasis, and defense against viral infections and mutations. In many human diseases, the genes controlling the apoptotic process are suppressed, over-expressed or altered by mutations [2]. Importantly, the vast majority of human cancers are associated with mutations in various apoptotic checkpoint proteins, or tumor suppressor genes. A preponderance of epidemiological and molecular evidence suggests that these mutations play a critical role in enabling the progression of malignancy through suppression of the apoptotic process [3].

The three fundamental steps of apoptosis are initiation, commitment, and execution [4]. Several apoptotic stimuli, such as irreparable DNA damage, have been shown to activate the cellular caspase enzymatic cascade pathway, which is a central part of apoptosis. It is believed that the subsequent proteolytic cleavage of a variety of intracellular substrates, such

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as poly(ADP-ribose) polymerase (PARP) and the retinoblastoma (Rb) protein, by effector caspases leads to the terminal hallmarks of apoptosis, such as nuclear blebbing [5–8].

Selective activation of apoptotic pathways in cancer cells, but not in normal ones, is currently being pursued as a novel strategy for cancer treatment. It has been shown that standard cytotoxic chemotherapeutic drugs induce apoptosis not only in malignant but also in normal cells, while more aggressive cancer cells become resistant. This narrow therapeutic window often necessitates aggressive dosing regimens which are frequently discontinued due to toxic side effects. Therefore, it is essential to develop targeted therapies that minimize toxic side effects by specifically targeting and destroying tumors without harming non-malignant tissue.

Small synthetic molecules with apoptosis-inducing ability have potential to be developed into novel chemotherapeutic drugs. These molecules can be easily synthesized and structurally manipulated for selective development [9]. For more than 60 years, β -lactam antibiotics have played an essential role in treating bacterial infections [10,11]. These β -lactam drugs selectively disrupt the formation of bacterial cell walls, while eukaryotic cells are not affected, making β -lactam therapy safe for the patients. Recently, a new class of *N*-thiolated β -lactams was found to inhibit growth in methicillin-resistant *Staphylococcus aureus* [12–15]. Subsequent work has demonstrated that these *N*-thiolated β -lactams are capable of inducing apoptosis in tumor cells, making them good candidates for anti-cancer drugs [9].

We have previously reported that β -lactam L-1 (Fig. 1A), the most potent of the *N*-thiolated β -lactams tested, induces DNA damage, inhibits DNA replication and activates the apoptosis in cultured human tumor cells in a time- and concentration-dependent manner [9]. We have also demonstrated that L-1 selectively induces apoptosis in leukemic Jurkat T over normal immortalized YT cells and that L-1 induces apoptosis in several solid human tumor cell lines [9]. However, the *in vivo* effect of these β -lactams has not been investigated yet.

In the current study, we report two major *in vivo* findings. First, by using subcutaneous xenografts of human breast cancer MDA-MB-231 cells implanted in nude mice, we show that L-1 inhibits tumor growth *in vivo* in a dose-dependent manner. In addition, we demonstrate that the anti-tumor activity of L-1 is associated with its abilities to induce DNA damage and apoptosis in tumor tissues, suggesting a potential molecular mechanism.

2. Materials and methods

2.1. Materials

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The APO-DIRECT kit for terminal deoxynucleotidyl transferase-mediated UTP nick-end labeling (TUNEL) was purchased from BD Biosciences (San Jose, CA, USA). Antibodies against actin, heat shock protein 70 (Hsp70), DNA damage inducible gene-45 (GADD45), anti-goat and anti-mouse IgG-horseradish peroxidase were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Mouse monoclonal antibody against human PARP was purchased from BIOMOL International LP (Plymouth Meeting, PA, USA).

2.2. Synthesis of β -Lactam 1

The *N*-thiolated β -lactam L-1 was synthesized using a procedure described previously [12,15].

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

Human Jurkat T cells were cultured in RPMI 1640 medium and human breast cancer MDA-MB-231 cells were grown in DMEM/F12 (1:1). Both media were supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin and maintained at 37 °C in a humidified incubator with an atmosphere of 5% CO₂. A whole cell extract was prepared as previously described [16]. For Western blot analysis, the cell or tissue extracts were separated by SDS-PAGE and transferred to a nitrocellulose membrane, followed by visualization using the enhanced chemiluminescence (ECL) reagent (Amersham Biosciences, Piscataway, NJ, USA).

2.4. Cellular proliferation assay

MTT assay was used to determine the effect of β -lactam L-1 on overall proliferation of breast cancer MDA-MB-231 cells. Cells were plated in a 96-well plate and grown to 70–80% confluency, followed by addition of L-1 at indicated concentrations for 24 h. MTT (1 mg/ml) in PBS was then added to wells and incubated at 37 °C for 4 h to allow for complete cleavage of the tetrazolium salt by metabolically active cells. Next, MTT was removed and 100 µl 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.

2.5. Human breast tumor xenograft experiments

Female athymic nude mice aged 5 weeks were purchased from Taconic Research Animal Services (Hudson, NY) and housed in accordance with protocols approved by the Institutional Laboratory Animal Care and Use Committee of Wayne State University. Human breast cancer MDA-MB-231 cells (5×10^6) suspended in 0.1 ml of serum-free RPMI 1640 were inoculated subcutaneously (s.c.) in both flanks of each mouse. When tumors reached sizes of $\sim 150 \text{ mm}^3$ in average, the mice were randomly grouped ($n = 7$) and treated by daily s.c. injection with either 20 or 30 mg/kg of L-1 or the solvent (PBS containing 1% DMSO). Tumor size was measured every other day using calipers and their volumes were calculated according to a standard formula: $\text{width}^2 \times \text{length}/2$. Mice were sacrificed after 29 days of treatment when control tumors reached $\sim 900 \text{ mm}^3$. The tumors were collected and the tumor tissues were used for different assays.

2.6. Terminal nucleotidyl transferase-mediated nick-endlabeling (TUNEL) assay and hematoxylin and eosin(H&E) staining

TUNEL assay using treated cells was performed with an APO-Direct kit to determine the extent of DNA strand breaks measured by flow cytometry according to the manufacturer's instructions. TUNEL assay using *in situ* apoptosis detection kit was performed as described previously [17]. H&E staining in tumor tissue was performed following manufacturer's protocols. Western blot analysis using animal tumor samples was performed similarly as described above using cultured cancer cells.

2.7. Statistical analysis

To evaluate the difference between the treatment and solvent control, the Student's *t* test was applied. Variance analysis of ANOVA (Statistical Computation DASC software) was used to test differences among multiple independent groups in the *in vivo* study. The statistical difference was indicated as $P < 0.05$ and significant difference as $P < 0.01$.

3. Results

3.1. Treatment with L-1 increases levels of GADD45 β and Hsp70 proteins in leukemia Jurkat T cells

We have previously reported that *N*-thiolated β -lactams have the ability to induce DNA strand breaks selectively in transformed and malignant, but not in non-transformed cells [18]. We hypothesized that β -lactam-induced DNA damage should induce expression of many genes that are involved in DNA repair. To test this hypothesis, we treated human leukemia Jurkat T cells with 60 μ mol/L of L-1 for various hours, followed by measuring protein levels of GADD45 and Hsp70, both of which play important roles in DNA damage repair and cellular stress [19–21]. Indeed, L-1 treatment increased the level of Hsp70 protein after 6 h, which was further increased afterwards (Fig. 1B). Similarly, L-1 treatment for 12 h significantly increased expression levels of GADD45 protein (Fig. 1C). As a control, apoptosis-specific PARP cleavage was detected after 12 h of treatment (Fig. 1B), while DNA strand breaks were induced by L-1 at as early as 2 h (76% of TUNEL-positive cells; Fig. 1D). These data suggest that L-1 induces DNA damage that then triggers induction of GADD45 and Hsp70 expression and apoptosis.

3.2. L-1 treatment increases levels of GADD45 β and Hsp70 proteins, associated with induction of apoptosis in cultured human breast cancer MDA-MB-231 cells

Our goal was to investigate the *in vivo* effect of L-1 in a human breast cancer MDA-MB-231 xenograft mouse model. We therefore first determined the effect of L-1 in cultured MDA-MB-231 cells. We tested the anti-proliferative potency of L-1, by plating the MDA-MB-231 cells in a 96-well plate and treating them with various concentrations of L-1 for 24 h, followed by an MTT assay. We found that L-1 inhibits proliferation of MDA-MB-231 cells in a dose-dependent manner (Fig. 2A); at 25 μ mol/L concentration L-1 inhibited cell proliferation by approximately 41% (P value < 0.01) whereas at 75 μ mol/L concentration it induced 80% inhibition (P value < 0.01). Inhibition of MDA-MB-231 cell proliferation by L-1 was associated with a dose-dependent PARP cleavage (Fig. 2B). These data show that the ability of L-1 to inhibit cell proliferation is associated with apoptosis induction not only in leukemia Jurkat T cells but also in the breast cancer MDA-MB-231 cell line. Importantly, under the same conditions, protein levels of Hsp70 and GADD45 in MDA-MB-231 cells were also increased in an L-1 dose-dependent manner (Fig. 2B), further supporting the DNA-damaging activity of L-1 in cultured breast cancer cells.

3.3. The growth-inhibitory, DNA-damaging and apoptosis-inducing effects of L-1 in human breast tumor xenografts

After demonstrating that L-1 could efficiently inhibit proliferation and induce apoptosis in cultured MDA-MB-231 cells, we investigated its anti-tumor activity *in vivo*. Human breast cancer MDA-MB-231 cells (5×10^6) were implanted s.c. in 5-week-old female athymic nude mice. Upon reaching a palpable size ($\sim 150\text{mm}^3$), the mice were daily s.c. injected with either 20 or 30 mg/kg of L-1, or solvent control for 29 days. At the end of the trial, the measurement of tumor size showed that tumor growth was inhibited by 32% and 63% in the mice treated with 20 and 30 mg/kg of L-1, respectively (Fig. 3A). The statistical analysis showed there were significant differences among the three groups (the solvent, low dose and high dose of L-1), and between each L-1 treatment and solvent groups as well. After a 29 day treatment, the averaged body weight of the mice in groups of the solvent, 20 and 30 mg/kg of L-1 treatment was 29.9, 29.7 and 29.4 g, respectively (no statistical difference observed; $P > 0.1$).

To investigate whether L-1 induces DNA damage *in vivo*, TUNEL assay was performed using formalin-fixed tumor tissue. DNA strand breaks, indicated by TUNEL positivity were observed in tumors from animals treated with L-1, but not the control (Fig. 3B).

We then investigated whether L-1-induced DNA damage is associated with increased levels of Hsp70 and GADD45 proteins. We extracted proteins from the tumor tissues for Western blot analysis. We found that induction of Hsp70 protein was most pronounced at 30 mg/kg treatment whereas induction of GADD45 protein occurred after both 20 and 30 mg/kg treatments (Fig. 3C).

The results from TUNEL assay (Fig. 3B) also indicate induction of apoptosis by L-1 in MDA-MB-231 tumors. To further confirm *in vivo* apoptosis induction, H&E staining was also performed using formalin-fixed tumor tissue. The apoptotic feature, condensed nuclei, was shown by H&E staining in the tumors from mice treated with L-1 (Fig. 3 B). Taken together, these data show that the L-1 has anti-breast tumor ability, associated with its ability to induce DNA damage and apoptosis in breast tumor tissues *in vivo*.

4. Discussion

Since many currently used anti-cancer therapies are toxic and eventually cause resistance to the treatment, researchers are attempting to search for new anti-cancer agents with little or no toxicity. It is generally believed that an important property of a potential anti-cancer drug is its ability to induce apoptosis, preferably in a highly selective manner in cancer over normal cells. It has been shown that antibiotic therapies typically use the unique molecular targets of microbes, which makes them generally safe for the patient. β -lactam antibiotics are powerful and potent inhibitors of bacterial growth [10] and interestingly, one group called *N*-thiolated β -lactams, possesses anti-proliferative activity in cultured human tumor cells [9]. Since they were also observed to have little to no effect on normal cells [18], their anti-cancer potential and expected lack of toxicity classified them as excellent candidates for anti-cancer drug development.

Our previous work with *N*-thiolated β -lactams demonstrated that these compounds acted through DNA damage that subsequently induced S-phase arrest, and apoptosis [9]. Apoptosis induced by these compounds is caspase-dependent and associated with cytochrome c release [9], and is selectively induced in cancerous over normal cells [18].

Our hypothesis that β -lactam-induced DNA damage induces expression of genes/proteins that are involved in DNA repair was supported by the increased levels of GADD45 and Hsp70 proteins in cultured Jurkat T and MDA-MD-231 cell lines (Figs. 1 and 2) and, more importantly, in MDA-MB-231 tumors treated with L-1 (Fig. 3). It has been well documented that both of GADD45 and Hsp70 proteins play important roles in DNA damage repair and cellular stress [19–21]. It has been reported that the heat-inducible Hsp70 is a survival protein that confers cytoprotection against a variety of death-inducing stimuli and increases the tumorigenicity of rodent cells [22]. It has also been demonstrated that Hsp70 stimulates the polymerase δ DNA base excision repair enzyme [20]. Therefore, it is possible that in a response to DNA damage induced by β -lactam L-1 treatment, Hsp70 protein translocates to the nucleus and gets involved in DNA damage repair by interacting with DNA, either directly or through binding partners. This possibility, however, needs further investigation.

A GADD45 family protein was shown to be involved in G₂/M cell cycle arrest following DNA damage, and in the regulation of S-phase checkpoint [21]. Moreover, GADD45 family proteins function as specific activators of MTK1, a MAPK kinase kinase upstream in the p38 pathway [23], and induce apoptosis [24]. It has been suggested that specific binding of GADD45 to p38 is a potential mechanism by which GADD45 has a direct role in p38

activation by Ha-Ras [25]. It was also reported that ectopic expression of GADD45 in AML12 cells is sufficient to activate p38 and to trigger apoptotic cell death, whereas antisense inhibition of Gadd45 expression blocks TGF- β -dependent p38 activation and apoptosis [21]. Furthermore, TGF- β was shown to activate p38 and induce apoptosis in mouse primary hepatocytes from wild-type mice, but not from Gadd45 knockout mice [21]. All of these findings suggest that GADD45 participates in TGF- β -induced apoptosis by acting upstream of p38 activation. Since we have previously identified p38 MAP kinase as a central player in β -lactam-induced apoptosis [9], our results here suggest that GADD45 is one of the targets for β -lactam L-1, which activates p38, thereby inducing apoptosis. In the near future, we plan to perform an experiment in which GADD45 gene expression will be blocked by using its siRNA in order to investigate the contribution of GADD45 to L-1-induced DNA damage, p38 activation, apoptosis induction and tumor growth inhibition.

It has not been elucidated so far whether β -lactams possess anti-tumor activity by *in vivo* study previously. Therefore, we tested the effects of β -lactam L-1 in mice bearing human MDA-MB-231 xenografts. Our data showed that treatment with β -lactam L-1 caused a significant inhibition of MDA-MB-231 tumor growth in nude mice (Fig. 3A). Importantly, the anti-tumor activity of β -lactam L-1 was associated with induction of DNA damage indicated by TUNEL positivity and increased expression of GADD45 and Hsp70 proteins in tumor tissue treated with L-1 (Fig. 3B and C). We conclude that induction of DNA damage and apoptosis *in vivo* contribute to the anti-tumor activity of the β -lactam L-1. Taken together, our findings from this *in vivo* study strongly suggest that *N*-thiolated β -lactams, such as L-1, possess significant potential as novel anti-cancer drugs.

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Fig. 1. Induction of apoptosis and increase of protein levels of Hsp 70 and GADD45 in human leukemia Jurkat T cells by *N*-thiolated β -lactam L-1 (L-1) treatment. (A) Chemical structure of L-1. (B) Jurkat T cells were treated with 60 μ mol/L of L-1 for indicated time points, followed by Western blot analysis using antibodies against Hsp70, PARP and β -actin. DMSO was used as a control. (C) Western blot analysis of Jurkat T cells treated with 60 μ mol/L of L-1 for 2 and 12 h, using antibodies against GADD45 and β -actin. (D) Jurkat T cells were treated for 2 and 12 h with 60 μ mol/L of L-1, followed by flow cytometry for TUNEL assay. DMSO was used as a solvent control.

Fig. 2. Inhibition of cell proliferation and induction of apoptosis in MDA-MB-231 cancer cells. (A) Highly metastatic and malignant breast cancer MDA-MB-231 cells were treated for 24 h with different concentrations of L-1 (25, 50, and 75 μmol/L). After 24 h, medium was removed, and cells were treated with MTT solution as described in Section 2. ** $P < 0.01$; Bars, SD, mean of three experiments. (B) Western analysis of samples prepared from MDA-MB-231 cells treated for 24 h as indicated.

Fig. 3.

The anti-tumor activity of L-1 is associated with induction of DNA damage and apoptosis *in vivo* measured by up-regulation of GADD45 and HSP70 proteins. Female nude mice bearing MDA-MB-231 tumors were treated with either control solvent (Sol) or L-1 at 20 or 30 mg/kg/d for up to 29 days. (A) Inhibition of MDA-MB-231 tumor growth by L-1. Points, mean tumor volume in each experimental group containing seven mice; SD, $**P < 0.01$. (B) TUNEL assay and H&E staining using mouse tumor samples. (C) Effects of L-1 at the end point of the experiment. Tumors were collected after 29 day treatment, and the prepared tissue proteins were analyzed by Western blot using antibodies against Hsp70, GADD45 and β -actin.