Lysocellin, a metabolite of the novel drug ‘alopestatin’, induces G1 arrest and prevents cytotoxicity induced by etoposide

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Abstract. We report here that lysocellin, a polyether antibiotic from a streptomycete, induces G1 phase arrest in human osteosarcoma MG63 cells. Lysocellin up-regulates p21WAF1/Cip1 and down-regulates cyclin D1 at the mRNA level. In addition, cyclin D1 is down-regulated by the proteasome-dependent signal pathway in MG63 cells. In drug combination studies, we found that lysocellin treatment weakened the cytotoxic activity of etoposide in MG63 cells using a colony-formation assay. To study the in vivo efficacy of lysocellin, we isolated a novel compound related to lysocellin from the same streptomycete, and found that the novel drug is converted to lysocellin in vivo and decreases etoposide-induced alopecia in a neonatal rat model. We raise the possibility that this novel drug, named ‘alopestatin’, may be a promising agent against alopecia.

Introduction

Chemotherapy-induced alopecia (CIA) causes distress in cancer patients, and it is considered very important to alleviate this side effect during the course of anti-cancer chemotherapy. So far, no satisfactory strategy for suppressing human CIA is available (1,2). Many anti-cancer agents target specific phases of the cell cycle and are therefore selectively toxic to cells undergoing division (3). Inhibition of cell-cycle progression has been shown to decrease the cytotoxic activity of drugs targeting proliferating cells (4). In other words, given that some chemotherapeutic agents preferentially kill proliferating cells, growth arresting drugs might be effective in preventing CIA.

Cell-cycle progression is controlled by cyclins, cyclin-dependent kinases (cdks) and cdk inhibitors at various biological checkpoints (5). Cyclin D, in association with cdk4 and cdk6, plays important roles in cell-cycle control at the G1/S boundary. As a major target of cyclin D/cdk4 and -6 complexes, retinoblastoma gene product (RB) plays a critical role in the cell-cycle progression from the G1 to S phase (5). RB exists in its unphosphorylated form, which can bind to the E2F transcription factor and inhibit its transcriptional activity during the G0 and early G1 phases. Once RB is phosphorylated by cyclin D/cdk4 and -6, genes whose products are necessary for the initiation of DNA replication are activated during the G1-S phases (5). Cyclin D1 is regulated at both the transcriptional and post-transcriptional levels. Activation of the ERK pathway increases cyclin D1 mRNA, and cyclin D1 protein turnover is regulated by phosphorylation-triggered, ubiquitin-dependent proteolysis (6-8).

p21WAF1/Cip1 is a well-known cdk inhibitor that belongs to the CIP/KIP family, which directly regulates the activity of multiple cdks. The expression of p21WAF1/Cip1 is up-regulated by p53 in response to DNA damage, leading to cell-cycle arrest at the G1 checkpoint (9). However, we have shown that its induction occurs via p53-independent mechanisms in p53-negative human cell lines, such as WiDr and MG63, derived from colon cancer and osteosarcoma, respectively (10).
We describe lysocellin, a divalent polyether antibiotic, and the newly discovered drug ‘alopestatin’, isolated from Streptomyces sp. NA34896. Lysocellin is well known as a carboxylic ionophore that has been shown to improve the performance of beef cattle fed diets based on silage or concentrate (11,12). We newly found that lysocellin induces G1 arrest in various cell lines, including human osteosarcoma MG63 cells. When investigating the molecules associated with the G1 arrest induced by lysocellin, we observed the up-regulation of p21WAF1/Cip1 and down-regulation of cyclin D1 after lysocellin treatment. We also found that the novel drug alopestatin, a prodrg of lysocellin, prevents etoposide-induced alopecia in neonatal rats.

Materials and methods

Microorganisms and isolation of lysocellin and alopestatin. The culture was the designated strain, Streptomyces sp. NA34896, which has been deposited at the National Institute of Bioscience and Human-Technology (Ibaraki, Japan), where it was assigned the number, FERM-P-17984. The whole broth from the fermentation of Streptomyces sp. NA34896 was extracted with methanol. The extract was filtered, concentrated, and partitioned between ethyl acetate and water. Condensed oily material from the organic layer was purified with column chromatography (silica gel and ODS) and two compounds, NK34896A and NK34896B, were obtained by recrystallization. We determined the structures, finding that NK34896A is identical to lysocellin and that NK34896B is novel, and termed it ‘alopestatin’.

Cell culture, cell proliferation studies and treatment with lysocellin. The human osteosarcoma cell line, MG63, was maintained in DMEM medium with 10% fetal bovine serum and incubated at 37°C in a humidified atmosphere of 5% CO2. For the cell proliferation studies, cells were plated at a density of 2x10^4 cells in 35 mm-diameter dishes. Twenty-four hours after cell plating, various concentrations of lysocellin were added to the culture medium. From the first day to the fourth day after plating, the number of viable cells was counted by the trypan-blue dye exclusion test.

Analysis of cell-cycle progression. MG63 cells were exposed to lysocellin for 24 h and harvested. Cells were treated with Triton X-100 and RNase, and nuclei were stained with propidium iodide before their DNA content was measured using a Becton-Dickinson FACScalibur (Becton-Dickinson, Franklin Lakes, NJ). At least 20,000 cells were counted. The ModFit LD V2.0 software package (Becton-Dickinson, San Jose, CA) was used to analyze the data.

RNA extraction and Northern blot analysis. Total RNA was isolated from cells using a Sepasol RNA isolation kit (Nacalai Tesque Inc., Kyoto, Japan), and 10 μg of total RNA per lane was used for Northern blot analysis. The probes for human p21WAF1/Cip1 cDNA (13) and human cyclin D1 cDNA, a kind gift from Dr Richard G. Pestell (14), were used. Northern blot analysis was performed, as previously reported (15). The relative band intensity was assessed by the densitometric analysis of digitalized autographic images using NIH Image software (Scion Corp., MD).

Protein extraction and Western blot analysis. MG63 cells treated with either 1.0 μg/ml lysocellin or vehicle at different time points were harvested. Cells were lysed by lysis buffer (50 mM Tris-HCl, pH 7.5, 1% SDS) for the detection of the retinoblastoma gene (RB) product, or by RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.25% sodium deoxycholate, 1 mM EDTA) for the detection of other proteins. Western blot analysis was performed, as previously reported (16). The antibodies against p15INK4b (C-20), p18INK4c (N-20), p19INK4d (C-20), p27Kip1 (C-19), cyclin A (C-19), and cyclin E (HE12) were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. The antibodies against p21WAF1/Cip1 and RB (PM-14001A) were purchased from BD PharMingen, San Jose, CA. The antibody against cyclin D1 (MBL, Nagoya, Japan), and the antibody against β-actin (Sigma, St. Louis, MO) were used as the primary antibodies.

Tumor cell colony-formation assay. Exponentially growing MG63 cells were seeded at 1x10^5 cells in 100 mm-diameter dishes and pretreated with lysocellin or equivalent DMSO for 24 h prior to 24-h exposure to cytotoxic agents. Then, the cells were rinsed, 1x10^5 cells were inoculated in 100 mm-diameter dishes with fresh medium, and they were allowed to grow for 8-10 days. Cells were stained with 0.1% crystal violet, and the number of colonies was counted. The survival rate was calculated as the number of colonies following drug treatment relative to untreated controls.

Animals. Male Sprague-Dawley rats and female CDF-1 mice were purchased from Charles River Japan Inc. (Kanagawa, Japan) and Japan S.L.C. Co., respectively. All animal experiments were conducted in accordance with the guidelines provided by the Institutional Animal Care and Use Committee of Nippon Kayaku Co., Ltd., Tokyo, Japan.

Administration and blood collection. The experiment was performed on female CDF-1 mice after overnight fasting. Alopestatin was administered orally at doses of 25, 90 and 135 mg/kg to each group of mice (n=3/time point/dose). At each sampling time (0.083, 0.25, 0.5, 1, 2, 4, 6 and 8 h at a dose of 25 mg/kg; 0.083, 0.5, 1, 2, 4 and 24 h at doses of 90 and 135 mg/kg), blood samples were collected from the axillary artery under diethyl ether anesthesia. Plasma was obtained by centrifuging at 2,000 x g for 10 min at 4°C and was stored at -20°C until analysis.

Sample preparation. To plasma samples (0.1 ml), the internal standard solution (1 μg/20 μl) and 1 ml of 0.1 M ammonium formate (pH 7.0) was added. After mixing, 1 ml of toluene was added, and then extraction was carried out. The organic layer was recovered, and evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was reconstituted in 0.1 ml of mobile phase and filtered. A portion of each sample was injected into the liquid chromatography-tandem mass spectrometry (LC-MS/MS).

LC-MS/MS. Alopestatin and lysocellin in plasma was detected by LC-MS/MS. The analytes were separated by reversed-phase HPLC (LC-10AD Systems, Shimadzu) using a SenshuPak PhD column (2x100 mm, 5 μm, Senshu Science Co., Ltd.) at a flow rate of 0.2 ml/min. The mobile phase was 60%...
control culture with equivalent DMSO (0.1%) (▲) (bars, SD) (n=3). *P<0.05. LC, lysocellin.

dna contents in MG63 cells. After incubation with or without 0.03, 0.1, (◆), 0.3 (▲), or 1.0 μg/ml (◊) was added (day 1), and cell growth was compared with the control culture with equivalent DMSO (0.1%) (●). Data are shown as means (bars, SD) (n=3). (B) Effects of lysocellin on the distribution of cellular nuclei were analyzed by flow cytometry. The means of experiments are shown (bars, SD) (n=3). *P<0.05. LC, lysocellin.

acetonitrile-40% 20 mM ammonium formate, pH 7.0. The entirety of the HPLC column effluent was introduced into the electrospray mass spectrometer. Mass spectrometric analyses were performed on a Sciex API300 triple quadrupole mass spectrometer (PE-Sciex) in the positive-ion mode using multiple-reaction monitoring (MRM).

Pharmacokinetic analysis. The concentration-time data were analyzed by a non-compartmental model using WinNonlin Pharsight. Maximum concentration (Cmax) and time to maximum concentration (Tmax) were the experimentally observed values following oral administration of alopestatin. The area under the plasma concentration-time curve (AUC) was calculated using the trapezoidal rule.

Neonatal rat model of chemotherapy-induced alopecia. Rat pups born on the same day were randomized at birth (10 pups per mom per cage) and housed with moms during the study period. For the etoposide model of CIA, rat pups received etoposide (Nippon Kayaku Co. Ltd.) at 13 days of age. Alopestatin was formulated in PEG400 (Nacalai Tesque) and applied orally at 80 mg/kg per application. Rat pups received alopestatin or PEG400 once a day from 8 days to 13 days of age and etoposide, 6 mg/kg i.p., 2 h after alopestatin or polyethylene glycol at 13 days of age. Etoposide-treated rats experienced whole-body alopecia by 21 days of age.

Results

Lysocellin induces growth arrest at the G1 phase of cell-cycle progression in MG63 human osteosarcoma cells. We examined the effect of lysocellin on the proliferation of MG63 cells. Fig. 1A shows the growth of MG63 cells in the presence or absence of various concentrations of lysocellin. Lysocellin inhibited the proliferation of MG63 cells in a dose-dependent manner. Lysocellin at ≥0.1 μg/ml significantly inhibited cell growth compared to the control, and 0.3-1.0 μg/ml lysocellin showed a cytostatic effect. To investigate its effect on the cell-cycle progression of MG63 cells, the DNA content was measured by flow cytometric analysis (Fig. 1B). Treatment with 0.1-1.0 μg/ml of lysocellin for 24 h significantly increased the percentage of the G1 population and significantly decreased the S phase cells. These data demonstrate that lysocellin arrests the cell cycle of MG63 cells at the G1 phase.

Lysocellin-induced cell-cycle arrest is associated with the up-regulation of p21WAF1/Cip1 and down-regulation of cyclin D1 expression. We investigated the mechanisms involved in G1 phase arrest. We then analyzed the expression of several proteins whose regulation is crucial for G1-S transition, following treatment with various concentrations of lysocellin. Western blotting results showed that lysocellin increased p21WAF1/Cip1 protein and decreased cyclin D1 protein in a dose-dependent manner compared with control cells (Fig. 2A). In contrast, there were no obvious changes in the expression of cyclin A, cyclin E, p15INK4b, p16INK4a, p18INK4c, p19INK4d and p27Kip1 in response to lysocellin treatment (data not shown). We could not detect the expression of p16INK4a due to its homozygous deletion in MG63 cells. Both the changes in p21WAF1/Cip1 and cyclin D1 following lysocellin treatment were obvious at ≥0.1 μg/ml. These data are consistent with the result of the growth-inhibitory effect of lysocellin (Fig. 1A).

Time course studies showed that the p21WAF1/Cip1 was up-regulated by ~4.0-fold at 12 h and ~1.9-fold at 48 h, and cyclin D1 was down-regulated by ~0.34-fold at 6 h and ~0.17-fold at 48 h after treatment with 1.0 μg/ml of lysocellin (Fig. 2B). Next, we tested whether lysocellin alters the phosphorylation of RB protein. We found that hyperphosphorylated forms of RB protein began to be converted into the hypophosphorylated form 24 h after lysocellin treatment (Fig. 2C), which followed the up-regulation of p21WAF1/Cip1 and down-regulation of cyclin D1.

Additionally, we examined whether the expression of p21WAF1/Cip1 and cyclin D1 is regulated at the mRNA level. Northern blot analysis showed that 24 h of exposure to lysocellin increased p21WAF1/Cip1 mRNA and decreased cyclin D1 mRNA in a dose-dependent manner compared with control cells (Fig. 3A). Both the changes in p21WAF1/Cip1 mRNA and cyclin D1 mRNA following lysocellin treatment were obvious at ≥0.1 μg/ml. Time course studies showed that p21WAF1/Cip1 mRNA was up-regulated by ~3.2-fold at 12 h and ~2.7-fold at 48 h, and cyclin D1 mRNA was down-regulated by ~0.76-fold at 12 h and ~0.45-fold at 48 h, after treatment with 1.0 μg/ml lysocellin (Fig. 3B).
Collectively, these results indicate that lysocellin modulates both p21\(^{WAF1/Cip1}\) and cyclin D1 at mRNA and protein levels and, subsequently, a hyperphosphorylated form of the RB protein is converted into a hypophosphorylated form in MG63 cells.

Lysocellin down-regulates cyclin D1 through the proteasome-dependent signal pathway. Although lysocellin suppressed cyclin D1 expression at both mRNA and protein levels, the degree of mRNA suppression after lysocellin treatment seemed to be less than that of protein (Figs. 2 and 3). We then tried to elucidate whether lysocellin affected cyclin D1 degradation. As cyclin D1 undergoes ubiquitin-dependent proteosomal degradation (8), we examined whether MG132, a proteasome inhibitor, could block the lysocellin-induced down-regulation of cyclin D1. Cells were cultured with 1.0 μg/ml lysocellin in the presence or absence of a proteasome inhibitor, MG132, for 6 or 24 h. Thereafter, whole-cell extracts were prepared and analyzed by Western blotting. LC, lysocellin; ppRB, hyperphosphorylated RB; pRB, hypophosphorylated RB.

Figure 2. (A) Lysocellin up-regulates the p21\(^{WAF1/Cip1}\) protein and down-regulates the cyclin D1 protein in a dose-dependent manner in MG63 cells. MG63 cells were treated with the indicated concentrations of lysocellin or with equivalent DMSO (0.1%) for 24 h, and the expression of different proteins was examined by Western blotting. β-actin was chosen as a loading control as indicated. (B) Lysocellin up-regulates the p21\(^{WAF1/Cip1}\) protein and down-regulates the cyclin D1 protein in a time-dependent manner in MG63 cells. MG63 cells were exposed either to DMSO alone (−) or to 1.0 μg/ml lysocellin (+). The expressions of the p21\(^{WAF1/Cip1}\) and the cyclin D1 proteins were analyzed by Western blotting. (C) Lysocellin converts the phosphorylation of the RB protein into the hypophosphorylated form. MG63 cells were exposed to either DMSO alone (−) or 1.0 μg/ml lysocellin (+), and then the expression of the RB protein was analyzed by Western blotting. LC, lysocellin; ppRB, hyperphosphorylated RB; pRB, hypophosphorylated RB.

Figure 3. (A) MG63 cells were treated with the indicated concentrations of lysocellin or with equivalent DMSO (0.1%). Total RNA was extracted after 24 h of exposure to lysocellin at indicated doses, and then the expression of mRNA was examined by Northern blotting. GAPDH was chosen as a loading control. (B) MG63 cells were exposed to either DMSO alone (−) or 1 μg/ml lysocellin (+), and total RNA was extracted at the indicated times after treatment with lysocellin. GAPDH was chosen as a loading control. LC, lysocellin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Figure 4. Lysocellin down-regulates cyclin D1 through the proteasome-dependent signal pathway. MG63 cells were cultured with 1.0 μg/ml lysocellin in the presence or absence of a proteasome inhibitor, MG132, for 6 or 24 h. Thereafter, whole-cell extracts were prepared and analyzed for cyclin D1 protein by Western blotting. β-actin was used as a loading control. LC, lysocellin.

6 and 24 h after the treatments. These results suggest that one of the mechanisms by which lysocellin down-regulates cyclin D1 is through the proteasome-dependent signaling pathway.

Pretreatment of MG63 cells with lysocellin decreased cell death induced by etoposide. We performed drug combination studies of lysocellin with cytotoxic agents in several cell lines. MG63 cells were pretreated with 1.0 μg/ml of lysocellin or equivalent DMSO for 24 h prior to 24-h exposure to etoposide.
Colony formation of the cells in the control dish was almost abolished when etoposide alone was applied. However, it was clearly restored when both lysocellin and etoposide were applied (Fig. 5). These results suggest that lysocellin reduces the cytotoxicity of etoposide in MG63 cells. Similar results were obtained using human colon cancer HT29 cells (data not shown).

Alopestatin prevents hair loss in a neonatal rat model of chemotherapy-induced alopecia (CIA). From the results above, we expected lysocellin to protect normal cells from the cytotoxic effects of chemotherapeutic agents in vivo. Unfortunately, we could not use lysocellin in vivo studies because its toxicity was so intense that oral uptake was fatal to rats. To study the in vivo efficacy of lysocellin, we found a novel polyether compound, alopestatin, with a structure closely related to lysocellin in a second fraction of the same column chromatography that yielded lysocellin. A set of two hydrogen bonds in the end of the lysocellin structure was dehydrated in order to make the ringed structure present in alopestatin (Fig. 6A). After the oral administration of 25 and 90 mg/kg alopestatin, lysocellin was detected as expected in the vascular system of rats (Fig. 6B and Table I). We therefore believe that alopestatin would work as a prodrug of lysocellin in vivo.

The experiments were performed in a neonatal rat model of chemotherapy-induced alopecia (CIA). Rat pups were treated with saline and alopestatin at 25 or 90 mg/kg. Alopestatin was administered orally to each group of mice (n=5/time point/dose). At each sampling time, blood samples were collected from the axillary artery under diethyl ether anesthesia. Plasma was obtained by centrifuging, and alopestatin and lysocellin in plasma were analyzed by the liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described in Materials and methods. Data are expressed as means ± SD (n=3).

### Table I. Pharmacokinetic parameters of alopestatin and lysocellin after a single oral administration of alopestatin to female CDF-1 mice.

<table>
<thead>
<tr>
<th>Dose of oral administration of alopestatin (mg/kg)</th>
<th>Cmax (μg/ml)</th>
<th>Tmax (h)</th>
<th>[AUC]0-inf. (μg·h/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>AS 1.961</td>
<td>0.083</td>
<td>1.04</td>
</tr>
<tr>
<td></td>
<td>LC 2.458</td>
<td>2</td>
<td>13.06</td>
</tr>
<tr>
<td>90</td>
<td>AS 2.230</td>
<td>0.083</td>
<td>12.56</td>
</tr>
<tr>
<td></td>
<td>LC 5.867</td>
<td>2</td>
<td>61.74</td>
</tr>
</tbody>
</table>

AS, alopestatin; LC, lysocellin. Data are calculated for mean plasma concentrations (n=3).
The amount of hair present on the rat scalp at day 8, which we termed ‘alopecia index’, was scored as follows: 0, 100% hair loss; 1, >90% hair loss; 2, 50-90% hair loss; 3, 10-50% hair loss; 4, <10% hair loss; 5, no hair loss. Mann-Whitney’s U test was used to assess significant differences between the drug-treated and vehicle-treated groups. PEG, polyethylene glycol; ETP, etoposide; AS, alopestatin. *p<0.02.

Discussion

Lysocellin is a divalent polyether antibiotic from a streptomyecete (11) and a carboxylic ionophore. When fed to growing cattle, lysocellin improves daily gain and feed efficiency (12). In the present study, we studied its effect on culturing human cells and found that lysocellin arrests the cell cycle of human osteosarcoma MG63 cells at the G1 phase and inhibits cell growth. We next found that lysocellin significantly reduces cyclin D1 and increases p21^WAF1/CIP1, while other cyclins or cdks inhibitors are not affected. As expected, a hyperphosphorylated form of the RB protein was converted into a hypophosphorylated form in MG63 cells. Collectively, our results raise the possibility that the modulation of cyclin D1 and p21^WAF1/CIP1 may contribute to the G1 phase arrest induced by lysocellin, but we cannot exclude the possibility that other genes may also be associated with the G1 arrest.

We then analyzed the molecular mechanisms of reduction in cyclin D1 expression by lysocellin, and found that there are at least two pathways. Firstly, we found that lysocellin suppresses cyclin D1 mRNA. We next examined the effect of lysocellin on cyclin D1 promoter activity by a luciferase assay, but could not detect the suppression of promoter activity following 24-h exposure to 1.0 μg/ml of lysocellin (data not shown). On the other hand, it is known that cyclin D1 is degraded through the ubiquitin-dependent proteosomal pathway (8). We found that lysocellin-induced down-regulation of cyclin D1 expression was restored by a specific proteasome inhibitor, MG132 (Fig. 4). In contrast, lysocellin did not down-regulate p27^KIP1 (data not shown), which is also known to be degraded by the ubiquitin-proteosome pathway (17), suggesting that the activation of the proteosomal pathway by lysocellin is specific for cyclin D1.

We next examined the mechanisms by which lysocellin up-regulates p21^WAF1/CIP1 expression. We found that lysocellin increased p21^WAF1/CIP1 mRNA in a dose- and time-dependent manner (Fig. 3). We therefore examined the effect of lysocellin on the promoter activity of a p21^WAF1/CIP1 promoter-luciferase fusion reporter plasmid, but we could not detect the up-regulation of p21^WAF1/CIP1 promoter (data not shown). We also measured the stability of p21^WAF1/CIP1 mRNA by monitoring the rate of p21^WAF1/CIP1 mRNA degradation where transcription was halted by actinomycin D treatment, but lysocellin treatment did not increase the stability of p21^WAF1/CIP1 mRNA (data not shown).

We then investigated the possibility of applying lysocellin in cancer therapy. Interestingly, we found that lysocellin decreases the cytotoxic effects of etoposide in a colony-formation assay using MG63 cells (Fig. 5). Additionally, we found that lysocellin effectively protects human colon carcinoma HT29 cells from both doxorubicin and etoposide (data not shown).

We have two hypotheses about the mechanism behind the protective effect of lysocellin. The first is through the inhibition of apoptosis by the induction of p21^WAF1/CIP1, which...
However, lysocellin itself was too toxic. From the above results, we predicted that lysocellin would weaken the side effects of anti-cancer drugs. Chemo-therapy-induced alopecia (CIA) is a frequent emotionally distressing side effect of cancer chemotherapy (21, 22), for which there is currently no effective preventive therapy (23, 24). CIA is thought to arise when anti-cancer drugs ablate the minimal antagonism to antitumor efficacy. We therefore suggest that alopestatin might be promising as a drug to prevent CIA.

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