Antitumor Activity of Actinonin in Vitro and in Vivo

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ABSTRACT
Actinonin, an antibiotic and CD13/aminopeptidase N (APN) inhibitor, has been shown to be cytotoxic to tumor cell lines in vitro. We investigated the antiproliferative effects of actinonin on human and murine leukemia and lymphoma cells. Actinonin inhibited growth of NB4 and HL60 human cell lines and AKR mouse leukemia cells in vitro with an IC50 of about 2–5 μg/ml. The inhibitory effect on CD13-positive cells was not blocked by pretreatment with the anti-CD13/APN monoclonal antibody F23, which binds with high affinity to the active site of CD13/APN and blocks its enzymatic activity. Moreover, F23 alone was not inhibitory to CD13-positive cells. Furthermore, a similar inhibitory IC50 of actinonin was seen in the CD13-negative cell lines RAJI and DAUDI human lymphoma. These data suggest that the inhibitory effect of actinonin is not mediated by inhibition of CD13/APN. Cell cycle analysis showed that actinonin induces a G1 arrest in HL60 and NB4 cells; apoptosis was observed in 20–35% of the cells as measured by intracellular flow cytometry. To assess whether these effects could be seen in vivo, the effect of actinonin on the syngeneic AKR mouse leukemia model was evaluated. Actinonin showed dose-dependent antitumor effects on AKR leukemia in vivo, resulting in a survival advantage. In conclusion, apoptosis, growth inhibition, and therapeutic effects in vivo are induced by actinonin and are not likely to be mediated by CD13/APN.

INTRODUCTION
CD13/APN2 (EC 3.4.11.2) is a ubiquitous cell surface zinc aminopeptidase involved in down-regulation of regulatory peptide signals (1). Recently, APN has been shown to be the major receptor for the enteropathogenic coronavirus TGEV (2) and for human coronavirus 229E (3) and to be involved in tumor cell invasion (4, 5). Human APN is identical to the myeloid differentiation antigen CD13 (6, 7) found on HL60 leukemia cells (7, 8), myeloid and monocytic cells, and most myeloblastic leukemias as well as on cells and tissues outside the hematopoietic system including fibroblasts, intestinal epithelium, renal tubular epithelium, and synaptic membranes of the central nervous system (1). APN occurs as a homodimer, and the molecule is a 150-kDa, transmembrane glycoprotein with an intracellular amino terminus (1). F23, an antihuman CD13/APN mAb, is able to completely block the active site of the enzyme (9).

Bestatin, a CD13/APN inhibitor, was examined in preclinical and clinical studies; bestatin could inhibit lymph node metastasis of P388 leukemia in mice (10) and was used in clinical trials in malignant skin tumors (11), in head and neck cancer (12), in esophageal cancer (13), and in gynecologic tumors (14). High doses of bestatin resulted in the significant inhibition of preexisting experimental and spontaneous metastasis in mice (15). Results with bestatin prompted us to examine the actions of actinonin as an anticancer agent. Actinonin, a naturally occurring antibiotic derivative of l-prolinol and a potent CD13/APN inhibitor, is obtained from the culture filtrates of a Streptomyces species classified as Streptomyces Cutere C/2 NCIB 8845 (16). Actinonin has been shown to be generally active against Gram-positive bacteria. The action of the antibiotic involves disruption of RNA synthesis in bacteria. In vivo, it has no apparent toxicity to mice in doses up to 400 mg/kg body weight (16). Actinonin is eight times more potent than bestatin (9).

We report here the antiproliferative effects of actinonin on human leukemia and lymphoma cells in vitro and the treatment of syngeneic AKR leukemia in an AKR mouse model using actinonin.

MATERIALS AND METHODS
Materials. Actinonin, amastatin, and bestatin were purchased from Sigma (St. Louis, MO). mAbs 4B4, OKT9, Leu-11a, Leu-15, Leu-M1, MY4, control IgG1, and IgG2a were purchased from Coulter (Hialeah, FL) or Becton Dickinson (San Jose, CA). Fluoresceinated, affinity-purified goat antiserum to mouse immunoglobulins (GAM-FITC) was purchased from Amgen (Thousand Oaks, CA). Stock vials of human IL-3 were stored at 4°C. For each experiment, all factors were diluted in serum-containing medium on the day of use.

Cell Separation Techniques. Bone marrow cells were obtained from healthy volunteers after informed consent. The mononuclear cells were isolated by centrifugation on Ficoll-Hypaque gradients (1.077 g/ml; Pharmacia Fine Chemicals, Piscataway, NJ), washed twice in PBS and suspended in Iscove’s modified Dulbecco’s medium containing 10% FCS (HyClone, Logan, UT) supplemented with penicillin (100 units/ml; Life Technologies, Inc.), streptomycin (100 μg/ml; Life Technologies, Inc.), and 3 mg/ml glutamine.
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CFU-GM. Low-density (1 × 10^5 cells/ml) or CD34+ (5 × 10^5 cells/ml) bone marrow cells were cultured in 35-mm tissue culture dishes (Corning, Corning, NY) in McCoy's modified assay medium containing 0.3% agar (DIFCO Laboratories, Detroit, MI) and 10% FCS (17). Cultures were stimulated by the addition of 100 ng/ml IL-3.

Animals. Five-week-old female AKR mice were purchased from Jackson Laboratory (Bar Harbor, ME). All bedding material was sterilized before use; the cages were covered with an air filter and maintained in isolation cabinets. Animal handling and experiments were performed in a sterile atmosphere using a laminar flow hood following institutional care guidelines.

Cell Lines and Culture Conditions. AKR leukemia cells were obtained from the spleen of old AKR mice who spontaneously developed leukemia at 10 months. HL60 (acute myeloid leukemia, CD13 positive), NB4 (acute promyelocytic leukemia, CD13 positive, from Dr. M. Lanotte of the Louis Pasteur Institute, Paris, France), and RAJI and DAUDI (B lineage Burkitt's lymphomas, CD13 negative) were maintained in culture using RPMI 1640 supplemented with 10% Serum Plus (JRH Biosciences, Lenexa, KS) and 10% heat-inactivated FCS (Intergen, Purchase, NY) at 37°C in a humidified atmosphere of 5% CO2. Cell viability was always higher than 90%, and cells were free of mycoplasma contamination.

Transplantation of AKR Leukemia Cells into AKR Mice and Therapy. A 0.1-ml aliquot containing 2 × 10^6 AKR leukemia cells from suspension culture was transplanted s.c. into AKR mice. Tumors grew s.c., and the cutaneous tumor size was measured as a cross product to derive surface area. To protect animals, mice were considered dead and sacrificed when the tumor surface area reached more than 400 mm^2. The test animals were treated i.p. with actinonin in a final volume of 0.1 ml. Control mice were treated with 0.1 ml of saline.

Flow Cytometric Analysis of CD11b and BCL-2 Proteins. Cells were incubated for 1 h on ice with phycoerythrin-conjugated anti-CD11b mAb (Becton Dickinson), washed with PBS and fixed in 2% paraformaldehyde for 10 min, and then exposed to 0.1% Triton X-100 for 10 min. After washing with PBS and blocking with 1% human AB serum, cells were incubated with FITC-conjugated anti-BCL-2 mAb (DAKO A/S, Carpinteria, CA) on ice for 30 min and then analyzed on an EPICS Profile II flow cytometer (Coulter). Ten thousand events were counted for each sample. Mean peak fluorescence intensity for an isotype-matched control antibody was set at 1.

Cell Cycle and Apoptosis Analysis by Flow Cytometry. Cells were collected and fixed in 1.5% paraformaldehyde and PBS for 15 min. After washing with PBS, the cells were resuspended in 70% ice-cold ethanol and kept at -20°C for up to 5 days. Analysis for cell cycle distribution and apoptosis was performed according to the instructions in the APOPTAG kit (Oncor, Gaithersburg, MD). Stained cells were analyzed on a FACSScan flow cytometer (Becton Dickinson); evidence of apoptosis and percentage of cells in each phase of the cell cycle was determined.
were analyzed by CellFIT and PC-LYSIS software (Becton Dickinson).

RESULTS

Actinonin Inhibited Growth of Leukemia Cells in Vitro. Actinonin was tested for its ability to kill CD13-positive and CD13-negative cells. Activity and cytotoxicity were determined by inhibition of incorporation of [3H]leucine into protein and by trypan blue exclusion. Dose-response curves were generated by testing the inhibitory effects of actinonin on the protein synthesis of NB4 and HL60 cells (CD13 positive) or RAJI cells (CD13 negative) in culture (Fig. 1A). In these in vitro studies, 2–5 μg/ml actinonin were found to be required to inhibit protein synthesis by 50% in CD13-positive and -negative cells.

The cytotoxicity of actinonin was initially determined by trypan blue exclusion (Fig. 1B and Table 1). The IC50 (the concentration of actinonin required to kill 50% of cells) ranged from 2–5 μg/ml, comparable to the concentration of actinonin required to inhibit 50% of protein synthesis. The similar dose-response curves for cytotoxicity and protein synthesis inhibition on cells that expressed or did not express CD13/APN suggested that the mechanism of cytotoxicity did not necessarily involve inhibition of CD13/APN by actinonin. Treatment of NB4 and HL60 cells for 4 days with 100 μg/ml mAb F23, which blocks substrate binding to CD13/APN and its activity (18), had no effect on cell viability (91% alive) in comparison to no treatment (90% alive) or an isotype-matched control antibody, TA99 (91% alive; data not shown). In addition, cytotoxicity of actinonin was not abrogated by pretreatment of cells with mAb F23, which blocks actinonin binding to its active site (19). These data together showed that the inhibition of cell growth by actinonin is not through the inactivation of cell surface enzyme.

Cell Cycle G1 Arrest and Apoptosis in Leukemia Cells during Actinonin Treatment. Cell cycle distribution of leukemia cells showed small changes early after treatment with 10 μg/ml actinonin. After 24 h of exposure to actinonin, the number of HL60 cells arrested in G1 phase increased, and the percentage of cells in S phase decreased (Fig. 2). A similar effect was seen in NB4 (G1 phase increased by 24%) and RAJI (G1 phase increased by 37%) after 24 h of treatment with 10 μg/ml actinonin (data not shown). This G1 arrest is in accordance with the growth inhibition observed after 2 days of exposure to actinonin (data not shown). Ninety-six h after exposure to actinonin at 10 μg/ml, 20–35% of HL60 and NB4 cells showed apoptosis, whereas only 10% of RAJI cells had apoptosis after treatment (Table 2).

The significant antiproliferative effects of actinonin in vitro and the previously published antiproliferative effects in vivo of bestatin, another APN inhibitor (1), prompted an analysis of the effects of actinonin in vivo against leukemia or lymphoma cells. To avoid the problems associated with a xenograft model, we chose to use a syngeneic leukemia/lymphoma model in AKR mice to better approximate actual human use. AKR cells are inhibited in vitro by actinonin with an IC50 of about 3 μg/ml (Table 1). In these experiments, AKR mice were injected s.c. with 2,000,000 AKR leukemia cells (day 0) and treated i.p. with injections of actinonin beginning at day 3.

The effect of actinonin on tumor growth and survival rates of AKR mice after transplantation of AKR leukemia cells was investigated. After transplantation, mice were treated with a total of five injections of actinonin (one injection daily for 5 days). As compared to controls, actinonin increased the mean survival time of mice by nearly twofold by reducing the rate of tumor growth. Reduction in tumor growth rates and prolongation of survival were actinonin dose-related (Fig. 3A). Toxicity due to actinonin was not observed. Actinonin doses up to 8000 μg/mouse (400 mg/kg) were tolerated without apparent toxicity (16).

In a second trial, mice were treated with 100 μg of actinonin daily for 3 days beginning at day 3 after transplantation and then treated by an additional three injections of actinonin (every other day). On day 17, the control mice showed tumors with a mean surface area of 287 ± 95 mm2. In contrast, no tumors were found in mice in the actinonin-treated group (Fig. 3B). These results indicate that actinonin has significant antitumor effects on AKR leukemia in vivo.

Table 1 Effect of actinonin on cell viability determined by trypan blue exclusion in vitro.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>IC50 (μg/ml)</th>
<th>Mean ± SD</th>
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<tbody>
<tr>
<td>HL60 cells</td>
<td>2 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>NB4 cells</td>
<td>5 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>DAUDI cells</td>
<td>2 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>RAJI cells</td>
<td>4 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>AKR cells</td>
<td>3 ± 0.6</td>
<td></td>
</tr>
</tbody>
</table>

*The IC50 is the concentration of actinonin required to kill 50% of cells.

Fig. 2 Effects of actinonin on cell cycle. Aliquots of HL60 cells were collected at different times after actinonin treatment (10 μg/ml). Cell cycle distribution was analyzed by flow cytometry as described in "Materials and Methods."

Table 2 Effect of actinonin (10 μg/ml) on apoptosis in cells at 96 h.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Control</th>
<th>Actinonin</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL60</td>
<td>2.9%</td>
<td>37.9%</td>
</tr>
<tr>
<td>NB4</td>
<td>9.1%</td>
<td>29.4%</td>
</tr>
<tr>
<td>RAJI</td>
<td>4.3%</td>
<td>13.6%</td>
</tr>
</tbody>
</table>

Effects of Actinonin on AKR Leukemia Cells in Vivo.

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In contrast, actinonin did not have any significant growth-inhibiting properties for s.c. implanted RAJI lymphoma in nude mice (data not shown) over the same dose range as was effective in the AKR model.

**Effects of Actinonin on Normal Human Bone Marrow Cell Growth ex Vivo.** The effects of actinonin, amastatin, and bestatin were evaluated on human bone marrow CFU-GM (Table 3). Actinonin decreased colony formation 16–56% in a dose-dependent manner. Bestatin (10 μg/ml) at doses designed to approximate its dose level in clinical trials (20), also decreased normal human bone marrow CFU-GM by 64–70%. This suggested that actinonin may have some myelosuppressive activities at high doses. F23 (antiactive site of CD3) and M195 (anti-CD33), both of which bind to bone marrow progenitors, had no effect on human bone marrow CFU-GM on days 7 and 14 (data not shown). These data further support the contention that the cytotoxic effects of actinonin are not mediated through CD13/APN.

**DISCUSSION**

Actinonin, a naturally occurring derivative of L-prolinol, is used as a potent inhibitor of CD13/APN. We report here: (a) the antiproliferative effects of actinonin on human leukemias and lymphoma cells in vitro; (b) the induction of growth arrest and apoptosis in target cells; (c) the antitumor effects of actinonin on AKR leukemias in AKR mice; and (d) that these effects do not seem to be mediated through inhibition of CD13/APN.

Actinonin had significant antiproliferative effects on human leukemias of various derivations. The cytotoxicity of actinonin was directly determined by trypan blue analysis and [3H]leucine incorporation. The IC50 was about 2–5 μg/ml. However, the IC50 for other CD13/APN inhibitors, amastatin and bestatin, was above 100 μg/ml (data not shown). Actinonin not only inhibited growth of CD13-positive cells (NB4 or HL60 cells) but also of CD13-negative cells (RAJI or DAUDI cells; Table 1), suggesting that the effect is not mediated by CD13/APN. Experiments with mAb F23 also suggested that the effect is not mediated by CD13/APN, because cell viability was not changed by treatment with mAb F23 in comparison to no treatment or treatment with an isotype-matched control antibody. In addition, the inhibitory effect of actinonin on CD13-positive cells was not blocked by pretreatment with the anti-CD13/APN mAb F23 (19). Because actinonin binds to F23 epitopes and is blocked by prior incubation with mAb F23 (18) and mAb F23 is not inhibitory of cell growth, we conclude here that actinonin-induced cell death in human leukemia cells is not likely to be associated with binding and inhibition of cell surface CD13/APN.

The effect of actinonin in vitro was mediated at least partly through G1 arrest and apoptosis. After 10 μg/ml actinonin treatment for 96 h, 20–35% of NB4 and HL60 cells showed evidence of apoptosis. The 1,10-phenanthroline, which inhibits APN activity by chelating the zinc ion (3, 21), also induces apoptosis in HL60 cells (22). Actinonin binds to zinc domains of CD13/APN, as determined by competition assays (18). This may suggest that the zinc-binding motif of important intracellular enzymes may account for apoptosis, but cell surface CD13/

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**Table 3** Response of low-density bone marrow cells to actinonin, amastatin, and bestatin.

Data represent the mean ± SD for four plates scored on day 7 and day 14. For these studies, 1 × 10³ low-density bone marrow cells were cultured for 7 and 14 days, respectively, with IL-3 (100 ng/ml).

<table>
<thead>
<tr>
<th>Drugs (doses)</th>
<th>No IL-3 (% of inhibition)</th>
<th>Day 7 (% of inhibition)</th>
<th>Day 14 (% of inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (saline)</td>
<td>0</td>
<td>177 ± 28</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>Actinonin (10 μg/ml)</td>
<td>0</td>
<td>99 ± 18 (44)</td>
<td>44 ± 8 (56)</td>
</tr>
<tr>
<td>Actinonin (5 μg/ml)</td>
<td>0</td>
<td>123 ± 10 (31)</td>
<td>49 ± 7 (51)</td>
</tr>
<tr>
<td>Actinonin (0.5 μg/ml)</td>
<td>0</td>
<td>148 ± 6 (16)</td>
<td>74 ± 7 (26)</td>
</tr>
<tr>
<td>Amastatin (5 μg/ml)</td>
<td>0</td>
<td>157 ± 1 (11)</td>
<td>82 ± 11 (18)</td>
</tr>
<tr>
<td>Bestatin (10 μg/ml)</td>
<td>0</td>
<td>63 ± 27 (64)</td>
<td>30 ± 7 (70)</td>
</tr>
</tbody>
</table>
APN does not seem to be associated with actinonin action. In addition, there is no increase in the percentage of CD11b in NB4, HL60, or RAJI cells (data not shown) after actinonin treatment, suggesting that there is no differentiating activity induced by actinonin.

Actinonin was also cytotoxic to RAJI cells, without an increase in apoptosis; in preliminary experiments designed to screen for other mechanisms or pathways involved in the anti-proliferative effects in these cells, BCL-2 expression was studied. BCL-2 expression, observed by intracellular flow cytometry in about 20% of HL60 cells and 60% of NB4 cells, showed no significant changes after treatment with actinonin. BCL-2 expression, however, was decreased by 80% in RAJI cells after treatment with actinonin. Other cell surface proteins (CD11b, 13, 15, 29, 33, and HLA-A) significantly decreased in NB4 cells after treatment with actinonin, consistent with the toxic effects of actinonin.

Bestatin, another APN inhibitor, has shown antitumor therapeutic effects in several clinical trials (23). In a multi-institutional study, 101 patients with acute nonlymphocytic leukemia were randomized to receive bestatin or control. The bestatin group achieved a statistically significant prolongation of both the remission duration and survival in patients aged 50–65 years (24). It also normalized the CD4:CD8 ratio in peripheral blood and maintained the immune homeostasis in cancer patients (25, 26). This may be beneficial to AIDS patients with lymphoma. Recently, bestatin also showed direct inhibition of the growth of human choriocarcinoma in nude mice in a dose-dependent manner (27). A recent randomized study in patients with non-Hodgkin’s lymphoma after autologous bone marrow transplant who were treated with bestatin showed increases in CD4 T-cell and B-cell numbers (26). Therefore, we investigated whether a more potent APN inhibitor, actinonin, could inhibit the growth of syngeneic AKR leukemia cells in a mouse model in vivo. Actinonin showed dose-dependent antitumor effects on AKR leukemia in vivo. There was a significant effect at doses of 100 μg/mouse (5 mg/kg). Prolonged treatment seemed to further improve the activity against the leukemias. Because actinonin shows no apparent toxicity in doses up to 400 mg/kg in mice (about 8 mg/mouse; Ref. 16) and no apparent toxicity was seen in the experiments conducted here, the drug may be considered safe in this mouse model at these doses. In contrast, little antitumor activity was seen in a nude mouse model. One explanation for this may be related to the hypothesis that these inhibitors work via a nonspecific immune augmentation in vivo (26, 28) that may not be possible in nude mice. Alternatively, there may simply be differences in the biology of the cells that account for the lack of effects with RAJI cells.

In conclusion, the data showed that actinonin induces G1 arrest and apoptosis in human leukemia and lymphoma cells; moreover, actinonin can treat AKR leukemia in AKR mice with minimal toxicity. The site of action does not seem to be via inhibition of CD13/APN. Details of the mechanisms of actinonin antileukemia and anti-lymphoma activity will require further study.

REFERENCES
Antitumor activity of actinonin in vitro and in vivo.
