Avicin D Selectively Induces Apoptosis and Downregulates p-STAT-3, bcl-2, and Survivin in Cutaneous T-Cell Lymphoma Cells

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Avicin D, a natural triterpenoid saponin, inhibits cell growth and induces apoptosis in transformed tumor cell lines in vitro and mouse skin carcinogenesis models in vivo. To investigate the anti-tumor effects of avicin D in cutaneous T-cell lymphomas (CTCL), we compared three CTCL cell lines and Sézary cells from three Sézary syndrome (SS) patients with normal CD4+ and activated CD4+ T cells from three healthy donors. Avicin D at 0.5–5 μg ml⁻¹ induced apoptosis in a time- and dose-dependent manner in three cell lines: MJ (−0.2 to 13% and 0.6–37%), Hut78 (2–39% and 3–53%), and HH (13–83% and 44–89%) at 24 and 48 hours, respectively. Avicin D at 0.5–5 μg ml⁻¹ for 48 hours caused more apoptosis in patients’ Sézary cells than in healthy donors’ CD4+ T cells and activated CD4+ T cells. The general caspase inhibitor Z-VAD-FMK and caspase-3 inhibitor Z-DEVD-FMK decreased avicin D-induced apoptosis in CTCL cells. Caspase-3 was activated and poly (ADP-ribose) polymerase was cleaved after avicin D treatment. Avicin D did not change the expression of signal transducer and activator of transcription-3 (STAT-3) but decreased phospho-signal transducer and activator of transcription-3 (p-STAT-3) protein levels in all three cell lines and two patients’ Sézary cells. Avicin D also decreased expression of the inhibitor of apoptosis protein survivin, the anti-apoptotic protein bcl-2, but not the pro-apoptotic protein bax in these CTCL cells. In summary, avicin D selectively induced apoptosis, inhibited STAT-3 activation, and decreased apoptosis inhibitors (bcl-2 and survivin) in CTCL cell lines and SS patients’ Sézary cells. Our findings underlie the therapeutic potential of avicin D in patients with SS.


INTRODUCTION

Cutaneous T-cell lymphomas (CTCL) are extranodal non-Hodgkin’s lymphomas with pleomorphic skin lesions and distinct T-cell markers. Mycosis fungoides (MF), the most common and indolent form of CTCL, is characterized by epidermotropic and malignant CD4+CD45RO+CLA+CCR+ helper/memory T cells, which may lack CD7 and/or CD26 expression. MF may evolve into a leukemic variant, Sézary syndrome (SS), or may transform to a large-cell lymphoma expressing CD30+ in some cases (Duvic and Cather, 2000; Kim et al., 2005). There are a limited number of Food and Drug Administration (FDA)-approved therapeutic modalities available to treat patients with MF/SS, including topical and oral bexarotene (Duvic et al., 2001a,b), intravenous denileukin diftitox (Olsen et al., 2001), photopheresis (Zic, 2003), and vorinostat (Duvic et al., 2007; Olsen et al., 2007). However, a number of non-approved agents are commonly used as a standard of care and include topical steroids and mustargen, phototherapy, IFNs, and chemotherapy (Duvic and Cather, 2000). Patients with refractory or transformed MF and SS have a poor prognosis and therapy is usually palliative. Alternative or complementary therapies, especially for advanced MF/SS, are needed.

Avicins are a family of natural triterpenoid saponins derived from the Cactus plant Acacia victoriae found in Australia’s deserts (Jayatilake et al., 2003). In vitro, avicins inhibit cell growth and induce apoptosis in leukemia and epithelial cancer cell lines (Haridas et al., 2001a, b; Mujoo et al., 2001). In a mouse skin carcinogenesis model, avicins have been shown to suppress both initiation and promotion phases of chemical carcinogenesis, as well as UV light B damage with resultant suppression of oxidative DNA and lipid damage (Hanausek et al., 2001; Haridas et al., 2004). Avicins have also reported to induce apoptosis by affecting mitochondrial function and activating the intrinsic caspase...
pathway (Haridas et al., 2001a, b; Mujoo et al., 2001). Avicins target and close the voltage-dependent anion channel, subsequently leading to lower cell energy metabolism and triggering cell apoptosis by permeabilization of the outer mitochondrial membrane and release of cytochrome c (Lemeshko et al., 2006; Haridas et al., 2007). Avicins also suppress multiple pro-inflammatory components of the innate immune system, including the transcriptional factor NF-κB (Haridas et al., 2001a, b), the phosphoinositide-3 kinase/AKT signaling pathway (Mujoo et al., 2005), as well as heat shock proteins (Gaikwad et al., 2005).

In this study, we evaluated the anti-tumor effects of avicin D on induction of apoptosis and modulation of signal transducer and activator of transcription-3 (STAT-3) and apoptosis-related proteins in CTCL cell lines and patients’ Sézary cells. Our data suggest that selective induction of tumor T-cell apoptosis, inhibition of STAT-3 activation, and downregulation of bcl-2 and survivin underlie the therapeutic potential of avicin D in patients with SS.

RESULTS
Avicin D treatment inhibited cell growth in CTCL cell lines
To determine whether avicin D inhibits CTCL cell growth, MJ, Hut78, and HH cells were treated with or without 0.5, 1, 2, and 5 μg ml⁻¹ avicin D for 24 and 48 hours, and their viability was evaluated by CellTiter 96 AQueous One Solution Cell Proliferation Assay. As the concentrations of avicin D increased from 0.5 to 5 μg ml⁻¹, HH cell growth was inhibited from 0 to 81% and from 24 to 88% at 24 and 48 hours, respectively, in a dose- and time-dependent manner compared with vehicle control (n = 3, P < 0.05). Similar results were also seen in MJ and Hut78 cell lines; however, MJ and Hut78 cells were less sensitive than HH cells to avicin D in inhibiting cell growth (Figure 1).

Avicin D treatment induced apoptosis in CTCL cell lines
To determine whether the growth inhibition of CTCL cells by avicin D results from apoptosis, MJ, Hut78, and HH cell lines were treated with or without 0.5, 1, 2, and 5 μg ml⁻¹ avicin D for 24 and 48 hours. As the avicin D concentration increased from 0.5 to 5 μg ml⁻¹, the number of HH cells staining for annexin V increased from 13 to 83% and 44 to 89% at 24 and 48 hours, respectively, in a dose- and time-dependent manner compared with vehicle control (n = 3; P < 0.05). Again, similar results were also seen in MJ and Hut78 cell lines; however, MJ and Hut78 cells were less sensitive than HH cells to avicin D in inducing apoptosis (Figure 2).

Avicin D treatment selectively induced apoptosis of SS patients’ Sézary cells
To confirm the results from cell lines, we also tested the pro-apoptotic effect of avicin D on primary Sézary cells from three SS patients who had 95–98% of circulating CD4⁺/CD26⁻ T cells compared with normal CD4⁺ T cells and activated CD4⁺ T cells from three normal donors (Table 1). As the avicin D concentration increased from 0.5 to 5 μg ml⁻¹, the number of SS patients’ Sézary cells stained for annexin V increased from 5.7 to 33% at 48 hours compared with vehicle control. As the avicin D concentration increased from 0.5 to 5 μg ml⁻¹, the number of healthy donors’ CD4⁺ T cells and activated CD4⁺ T cells stained for annexin V increased from −0.9 to 2% and 0.2 to 5.8% at 48 hours compared with vehicle control, respectively. Thus, avicin D induced more apoptosis in SS patients’ Sézary cells than in healthy donors’ controls (P < 0.05) (see Table 1). The results suggest that avicin D was selective in inducing apoptosis of SS patients’ Sézary cells relative to healthy donors’ controls.

Avicin D-induced apoptosis was dependent on caspase-3 in CTCL cells
Caspase-3 is a key downstream target for both the intrinsic and extrinsic apoptosis pathways (Porter and Jänicke, 1999). To determine whether caspase-3 activation is involved in avicin D-induced apoptosis, we examined the effects of the general caspase inhibitor Z-VAD-FMK and caspase-3 inhibitor Z-DEVD-FMK in CTCL cells. Both Z-VAD-FMK and Z-DEVD-FMK decreased avicin D-induced apoptosis in three CTCL cell lines and one patient’s Sézary cells (Figure 3). Furthermore, we checked the expression of pro-caspase-3, cleaved caspase-3, and poly (ADP-ribose) polymerase (PARP)
proteins in three CTCL cell lines (MJ, Hut78, and HH) and two SS patients’ Sézary cells compared with healthy donors’ CD4+ T cells. Western blotting showed that avicin D treatment activated caspase-3 and cleaved PARP as determined by the appearance of cleaved 17- and 19-kDa and 85-kDa fragments in CTCL cell lines and patients’ Sézary cells but not in healthy donors’ CD4+ T cells, respectively (Figure 4).

Avicin D treatment modulated apoptosis-associated proteins in CTCL cells

Constitutive activation of STAT-3 has been demonstrated to contribute to oncogenesis by stimulating cell proliferation and preventing apoptosis in CTCL (Nielsen et al., 1997; Eriksen et al., 2001; Sommer et al., 2004). To determine whether STAT-3 signaling and other apoptosis-related proteins are involved in avicin D-induced apoptosis, we examined the expression of STAT-3/phospho-signal transducer and activator of transcription-3 (p-STAT-3), bcl-2/bax, and survivin in three CTCL cell lines (MJ, Hut78, and HH) and two patients’ Sézary cells compared with healthy donors’ CD4+ T cells. Western blotting showed that p-STAT-3 protein expressed and decreased in three cell lines and two patients’ Sézary cells but not in healthy donors’ CD4+ T cells after avicin D treatment. Avicin D treatment did not significantly change protein levels of STAT-3 in all cell lines, patients’ Sézary cells, and healthy donors’ CD4+ T cells after avicin D treatment. The levels of pro-apoptotic protein bax remained constant in all cell lines, patients’ Sézary cells, and healthy donors’ CD4+ T cells after avicin D treatment. The levels of pro-apoptotic protein bcl-2 decreased after avicin D treatment in these cell lines and patients’ Sézary cells, but did not significantly change in healthy donors’ CD4+ T cells. Moreover, avicin D treatment decreased protein levels of survivin in these cell lines and patients’ Sézary cells. Survivin was not observed in healthy donors’ CD4+ T cells without or with avicin D treatment (Figure 4).
The autophagy inhibitor protected avicin D-induced cell death in CTCL cells

Because general caspase inhibitor Z-VAD-FMK and caspase-3 inhibitor Z-DEVD-FMK only partially decreased avicin D-induced apoptosis in CTCL cells, we hypothesized that avicin D may also induce a caspase-independent programmed cell death, such as autophagy. To test whether avicin D induces autophagy, three CTCL cell lines (MJ, Hut78, and HH) were pretreated with chloroquine (10 μM), an inhibitor of autophagy (Xu et al., 2007), for 6 hours and then incubated with avicin D (5 μM) for 48 hours. Our results showed that CTCL cells pretreated with chloroquine had improved cell viability (Figure 5a) and decreased cell death (Figure 5b) relative to cells treated with avicin D alone, suggesting that avicin D may also induce autophagy in CTCL cells.

DISCUSSION

Apoptosis plays important roles in embryogenesis, aging, and cancer cell survival (Webb et al., 1997). A major goal in clinical cancer research is to discover compounds that selectively induce apoptosis without adversely affecting normal cell growth. The anti-tumor actions of many chemotherapeutic agents have been attributed to the induction of apoptosis (Hickman, 1992). The most effective treatments for MF/SS, such as phototherapy (Baron and Stevens, 2003), photopheresis (Zic, 2003), bexarotene (Zhang et al., 2002), and vorinostat (Zhang et al., 2005), induce apoptosis of T cells. In this study, we show for the first time that avicin D, a novel triterpenoid saponin, selectively causes apoptosis of CTCL cell lines and patients’ Sézary cells compared with normal CD4+ and activated CD4+ T cells from healthy donors. Furthermore, recent studies showed that avicin D also induces autophagy, another type of programmed cell death (Xu et al., 2007). Of interest, our preliminary data revealed that CTCL cells pretreated with chloroquine, an inhibitor of autophagy, had reduced cell death and improved cell viability relative to cells treated with avicin D alone.
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in CTCL cell lines. Representative mean ± SD of triplicate determinations.

Figure 4. Effect of avicin D on modulation of apoptosis-associated proteins in CTCL cell lines and patients’ Sézary cells. Three cell lines (MJ, Hut78, and HH), Sézary cells from two SS patients, and normal CD4+ T cells from two healthy donors were treated with avicin D at 2 μg/ml for 24 hours. Cytoplasmic (10 μg) or nuclear (5 μg) proteins were fractionated by 8–12% SDS-PAGE, transferred onto nitrocellulose membranes, and subjected to the ECL detection analysis. The equivalent loading of proteins in each well was confirmed by actin and Ponceau staining.

Figure 5. The autophagy inhibitor protected avicin D-induced cell death in CTCL cell lines. Cells (1 x 10^5) were pretreated with or without 10 μM chloroquine for 6 hours and followed with avicin D (5 μg/ml) for 48 hours. Cell viability and cell death were measured by the CellTiter 96 AQueous One Solution Cell Proliferation Assay and Annexin V-FITC/PI Detection Kit. a) Effect of chloroquine on avicin D-induced cell growth inhibition; b) effect of chloroquine on avicin D-induced cell death. Each bar represented mean ± SD of triplicate determinations.

suggesting that avicin D may also induce autophagy in CTCL. Further experiments for detecting autophagosomes are needed to confirm our above observation in CTCL.

Avicins have been shown to induce apoptosis by affecting mitochondrial function and activating caspase-3 in the human T-cell leukemia Jurkat cell line (Haridas et al., 2001a,b). Caspase-3 is a key component of apoptosis, is activated in apoptotic cells, and cleaves cellular proteins, including PARP (Porter and Jánicek, 1999). Cleavage of PARP is a hallmark of apoptosis induced by various anti-tumor agents (Duriez and Shah, 1997). We have previously reported that caspase-3 is activated and PARP is cleaved in apoptotic CTCL cells after treatment with the retinoid X receptor (RXR)-selective retinoid bexarotene (Zhang et al., 2002) and with the histone deacetylase inhibitor vorinostat (Zhang et al., 2005). Of interest, another triterpenoid compound, CDDO (2-cyano-3-3, 12-dioxoolean-1, 9-dien-28-oic acid), also induces T-cell apoptosis in CTCL cells in association with activation of caspase-3 and cleavage of PARP (Zhang et al., 2004). In this study, we show that general caspase inhibitor Z-VAD-FMK and caspase-3 inhibitor Z-DEVD-FMK decreased avicin D-induced apoptosis in CTCL cells. Avicin D treatment also activated caspase-3 and cleaved PARP in three CTCL cell lines and patients’ Sézary cells but not in healthy donors’ CD4+ T cells. Thus, avicin D-induced apoptosis is at least partially dependent on caspase-3 in CTCL cells.

To understand the mechanism of apoptosis induced by avicin D, we examined the STAT-3 signaling pathway. STAT-3 is activated by a wide variety of extracellular and intracellular stimuli, including IL-2, IL-6, IL-7, IL-9, IL-10, and IL-15, and by intracellular tyrosine kinases, such as src and abl (Calo et al., 2003). After activation by phosphorylation, STAT-3 proteins dimerize and translocate from cytoplasm to the nucleus where they bind to specific DNA promoter sequences and regulate gene expression (Calo et al., 2003). Constitutive activation of STAT-3 contributes to oncogenesis by stimulating cell proliferation and preventing apoptosis in solid tumors and hematological malignancies, including CTCL (Sommer et al., 2004; Turkson, 2004; Mitchell and John, 2005). In particular, STAT-3 activation is possibly involved in the neoplastic transformation of CTCL and may, therefore, be a molecular target for therapeutic interventions, especially for advanced patients (Sommer et al., 2004). In this study, activation of STAT-3 was not observed in healthy donors’ CD4+ T cells, which is consistent with the previous report (van Kester et al., 2008). Furthermore, we
found that avicin D at concentrations causing apoptosis decreased expression of p-STAT-3 (but not STAT-3) in three CTCL cell lines and two patients’ Sézary cells. To our knowledge this is previously unreported. Thus, down-regulation of p-STAT-3 protein may be involved in avicin D-induced apoptosis of CTCL cells. However, the mechanisms of reducing p-STAT-3 by avicin D remain to be evaluated in CTCL.

The balance between bcl-2 and bax expression is critical in controlling caspase activation by regulating the release of cytochrome c from mitochondria (Marion and Hockenberg, 2003). Bcl-2 expressed in CTCL cells may increase survival and resistance of CTCL cells against radiotherapy and extracorporeal photochemotherapy (Osella-Aate et al., 2001; Breuckmann et al., 2002; Zhang et al., 2003). In this study, we show that avicin D at concentrations causing apoptosis did not change pro-apoptotic protein bax but decreased anti-apoptotic protein bcl-2 in CTCL lines and patients’ Sézary cells. Thus, alteration of the bcl-2/bax ratio is likely to be involved in avicin D-induced apoptosis of CTCL cells.

Survivin is a member of the inhibitor of apoptosis protein family (Ambrosini et al., 1997). Survivin is not expressed in normal adult tissues, but is abundantly expressed in fetal tissues, transformed cell types, and a variety of human tumors, including highly malignant non-Hodgkin’s lymphoma (Ambrosini et al., 1997; Adida et al., 1998). In vitro, survivin is not expressed in resting T cells but expressed in activated T lymphocytes, and its expression correlates with apoptosis resistance after lymphocyte activation (Kornacker et al., 2001). Survivin suppresses caspase activity and protects cells from apoptosis induced by a variety of agents (LaCasse et al., 1998). We previously demonstrated that CTCL cells express survivin protein, and its expression decreases after hexabrotenone treatment (Zhang et al., 2002). In this study, our results show that survivin was expressed in SS patients’ Sézary cells but not in healthy donors’ CD4+ T cells, which is consistent with the previous studies (Kornacker et al., 2001; Sors et al., 2008). Avicin D treatment decreased the protein levels of survivin in CTCL cell lines and in patients’ Sézary cells. This suggests that downregulation of survivin may be involved in caspase-3 activation in avicin D-induced apoptosis of CTCL cells.

In conclusion, avicin D selectively induced apoptosis of CTCL cell lines and Sézary cells from SS patients compared with normal CD4+ and activated CD4+ T cells from healthy donors. Avicin D-induced apoptosis was at least partially dependent on caspase-3 and was associated with downregulation of p-STAT-3 and anti-apoptotic proteins (bcl-2 and survivin). Although avicin D has not been used in patients yet, two other triterpenoid compounds (CDDO and CDDO-Me) are currently well tolerated in phase I clinical trials for the treatment of leukemia and solid tumors (Hong et al., 2007; Liby et al., 2007). Our findings provide a rationale for studying avicin D in patients with SS. Further experiments are needed to profile toxicity of avicin D and to better clarify the molecular details of anti-tumor effect for pre-clinical development.

**MATERIALS AND METHODS**

**Reagents**

Avicin D was isolated from the seedpods of the Australian desert plant, *Acacia victoriae* (Jayatilake et al., 2003). Avicin D was dissolved in distilled water to a stock concentration of 1 mg ml⁻¹ and stored at 4 °C. Serial dilutions (0.5, 1, 2, and 5 μg ml⁻¹) were freshly made in RPMI 1640 medium (Sigma-Aldrich, St Louis, MO). Z-VAD-FMK and Z-DEV-D-FMK were purchased from R&D Systems (Minneapolis, MN), dissolved in DMSO to a stock concentration of 20 mM, and stored at -80 °C. Monoclonal mouse anti-CD3 (clone UCHT1) was purchased form BioLegend (San Diego, CA). Chloroquine was purchased from Sigma-Aldrich.

**Cells and cell culture**

The human CTCL cell lines MI (G11), Hut78, and HH, obtained from American Type Culture Collection (Rockville, MD), were derived from peripheral blood of patients with MF, SS, and non-MM/SS aggressive CTCL, respectively (Gootenberg et al., 1981; Popovic et al., 1983; Starkebaum et al., 1991). Samples of peripheral blood were obtained for *in vitro* studies from three healthy donors and three SS patients with 95–98% circulating CD4+CD26− tumor T cells (Bernengo et al., 2001) (see Table 1). Samples were obtained during routine diagnostic assessments. The institutional review board of MD Anderson Cancer Center approved this study and the participants gave written informed consent. This study was conducted according to the Declaration of Helsinki Principles. Peripheral blood mononuclear cells from these healthy donors and patients were isolated by Vacutainer CPT (Becton Dickinson, San Jose, CA). CD4+ T cells were sorted by Human CD4 T Cell Enrichment Cocktail (StemCell Technologies, Vancouver, BC, Canada) according to the manufacturer’s instructions. CD4+ T-cell activation was performed using anti-CD3 antibody (clone UCHT1) as previously described (Ni et al., 2005). Cells were grown in RPMI 1640 medium (Sigma Chemical Co., St Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT), 2 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 1% penicillin–streptomycin in a humidified atmosphere with 5% CO₂ at 37 °C.

**Cell viability**

Cell viability was measured by CellTiter 96 AQuueous One Solution Cell Proliferation Assay (Goodwin et al., 1995) according to the manufacturer’s instructions (Promega, Madison, WI). Aliquots of 5 × 10⁴ cells per well were distributed in 96-well microplates (Falcon, Franklin Lakes, NJ) in 100 μl of medium and incubated with 0.5, 1, 2, and 5 μg ml⁻¹ avicin D for 24 and 48 hours, then 20 μl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt was added to each well and incubated for an additional 4 hours. The relative cell viability was determined at 490 nm using 96-well plate reader. Each experiment was performed in triplicate, and repeated a minimum of three times.

**Annexin V binding staining**

Analysis of annexin V binding was carried out with an Annexin V-FITC Detection Kit I (Pharmingen, San Diego, CA) according to the manufacturer’s instructions. Briefly, three cell lines (M, Hut78, and HH), CD4+ T cells, activated CD4+ T cells from three healthy...
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Isolation of cytoplasmic and nuclear extracts

Isolation of proteins was conducted as previously described (Zhang et al., 2002). Briefly, cells (5 x 10^6) were washed with ice-cold phosphate-buffered saline, harvested into 1 ml of phosphate-buffered saline, pelleted in a 1.5-ml microcentrifuge tube, and suspended in 400 µl of buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 1 mM EDTA, 1 mM EGTA (ethylene glycol bis[b-aminoethyl]ether)-N,N,N',N'-tetraacetic acid), 1 mM dithiothreitol, and 1 x protease inhibitor cocktail “complete mini” (Roche, Indianapolis, IN). After 20 minutes incubation on ice, the mixture was treated with a 24-G syringe needle and then centrifuged briefly to obtain the cytoplasmic supernatant. The nuclear pellet was resuspended in 40–80 µl of buffer C (10 mM HEPES (pH 7.9), 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 10% glycerol, and 1 x protease inhibitor cocktail “complete mini” (Roche)) and incubated at 4°C for 15 minutes with shaking. Protein concentrations were determined by the Bradford dye-binding protein assay (Bio-Rad, Richmond, CA) using BSA as a standard.

Western blot analysis

Western blotting was performed as previously described (Zhang et al., 2002). Briefly, cytoplasmic (10 µg) or nuclear (5 µg) proteins were subjected to SDS-PAGE (8-12%) electrophoresis and transferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). The membranes were blocked in 3% powdered milk in TBST (50 mM Tris (pH 7.5), 150 mM NaCl, 0.05% Tween 20) for 1 hour at room temperature, incubated with primary antibodies overnight at 4°C in 3% powdered milk in TBST, and washed extensively with TBST. They were incubated with 1:5,000 peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA) for 1 hour at room temperature. Monoclonal mouse anti-STAT-3 (1:1,000) antibody was obtained from BD Bioscience (San Diego, CA). Monoclonal mouse anti-bcl-2 (1:2,000), anti-survivin (1:500), anti-actin (1:12,000), anti-caspase-3 (1:1,000), and anti-PARP (1:1,000) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal rabbit anti-bax (1:500) antibody was obtained from Santa Cruz Biotechnology. Monoclonal mouse anti-phospho-STAT-3 (p-STAT-3) (1:1,000) and rabbit anti-cleaved caspase-3 (1:1,000) antibodies were obtained from Cell Signaling (Beverly, MA). Immunoreactive bands were visualized by enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, UK), and equivalent loading of proteins in each well was confirmed by actin and Ponceau staining.

Statistical analysis

Statistical significance of differences observed in cell viability and apoptosis in avicin D-treated cells compared with the untreated cells was determined by using the Student’s t-test. Experiments were repeated twice and carried out in triplicate. The minimum level of significance was a P<0.05.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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