Suberoylanilide hydroxamic acid (SAHA), an orally administered inhibitor of histone deacetylases, is currently in phase II clinical trials for cutaneous T cell lymphomas (CTCL), but the mechanism of SAHA action is unknown. In this study, we investigated the anti-tumor effects of SAHA in CTCL cell lines and freshly isolated peripheral blood lymphocytes (PBL) from CTCL patients with high percentage of circulating malignant T cells. Three cell lines (MJ, Hut78, and HH) and PBL from 11 patients and three healthy donors were treated with SAHA (1, 2.5, and 5 μM) for 24 and/or 48 h. Apoptosis was determined by flow cytometry analysis of sub-G₁ hypodiploid nuclei and/or annexin V binding populations. Acetylated histones and apoptosis-associated proteins were detected by Western blotting. SAHA at 1–5 μM for 24 and 48 h induced apoptosis in a concentration- and time-dependent manner in three cell lines: MJ (0%–7% and 1%–32%), Hut78 (4%–36% and 5%–54%), and HH (4%–67% and 8%–81%). SAHA at 1–5 μM for 48 h also induced more apoptosis of patients’ PBL than healthy donors’ (15%–32% versus 6%–13%, p < 0.05). SAHA treatment caused an accumulation of acetylated histones (H2B, H3, and H4), an increase of p21WAF1 and bax proteins, a decrease of Stat6 and phospho-Stat6 proteins, and activation of caspase-3 in CTCL cells. Our data suggest that selective induction of malignant T cell apoptosis and modulation of acetylated histones, p21WAF1, bax, Stat6, and caspase-3 may underlie the therapeutic action of SAHA in CTCL patients.

Key words: apoptosis/cutaneous T cell lymphoma/histone deacetylase inhibitor


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 malignant CD4⁺ CD45RO⁺ CLA⁺ CCR7⁺ T cells that also lack CD7 and/or CD26. MF may evolve into a leukemic variant, Sézary syndrome (SS), or transform to large cell lymphomas; Hodgkin’s lymphomas with pleomorphic skin lesions and thereby alter the expression of genes regulating cell survival, proliferation, differentiation, and apoptosis (Rosato and Grant, 2003; Mei et al, 2004; Drummond et al, 2005). Suberoylanilide hydroxamic acid (SAHA), an inhibitor of class I and II HDAC, induces cell growth arrest and apoptosis of a broad spectrum of transformed cells in vitro and in vivo studies (De Ruiter et al, 2003; Marks et al, 2003; Mei et al, 2004; Drummond et al, 2005). SAHA is currently in phase I and II clinical trials for the treatment of various cancers and has shown promising anticancer activity at doses that are well tolerated by patients (Kelly et al, 2003; Piekarz and Bates, 2004). Clinical evaluation of SAHA in patients with CTCL has revealed that oral SAHA produces clinically meaningful partial responses in ten of 37 advanced, heavily pretreated patients associated with decreased pruritus.¹

SAHA’s mechanism of action in CTCL has not yet been demonstrated. In this study, we performed in vitro translational studies to further define the anti-tumor effects of SAHA in well-established CTCL cell lines and freshly isolated peripheral blood lymphocytes (PBL) from SS/MF patients with high percentage of circulating malignant T-cells (Bernengo et al., 2001).

Results

SAHA inhibited cell growth of CTCL cell lines To determine whether SAHA inhibits CTCL cell growth, MJ, Hut78, and HH cell lines were treated with or without SAHA (1, 2.5, and 5 μM) for 24 and 48 h, and their viability was evaluated by CellTiter 96 AQ One Solution Cell Proliferation Assay. As the dose of SAHA increased from 1 to 5 μM over 24 and 48 h, growth inhibition of HH cells increased from 2% to 34% and from 21% to 70% in a concentration- and a time-dependent manner compared to vehicle control (n = 3, p < 0.05). Similar results were also seen in the MJ and Hut78 cell lines; however, MJ cells were less sensitive to SAHA (Fig 1A). These results demonstrated that SAHA is an inhibitor of CTCL cell line growth in vitro.

SAHA selectively induced apoptosis of CTCL cell lines and patients’ PBL To determine whether growth inhibition of SAHA is due to cell-cycle arrest and/or to apoptosis in CTCL cell lines, MJ, Hut78, and HH cells were treated with or without SAHA (1, 2.5, and 5 μM) for 24 and 48 h. As the dose of SAHA increased from 1 to 5 μM over 48 h, the percent of HH cells with sub-G₁ population increased from 11% to 88% in a concentration-dependent manner compared to vehicle control. As the incubation time of SAHA at 2.5 μM increased from 24 to 48 h, the percent of HH cells with sub-G₁ population increased from 37% to 80% in a time-dependent manner compared to vehicle control (Fig 1B). Similar results were also seen in the MJ and Hut78 cell lines (Fig 1C). An increased percentage of cells in the sub-G₁ population was accompanied by a loss of cells in the G₁, S, and G₂/M phases, suggesting cells are undergoing apoptosis (Zhang et al., 2002).

In parallel to the cell cycle analysis, we also investigated the effect of SAHA on annexin V binding in three CTCL cell lines. As the SAHA concentration increased from 1 to 5 μM for 24 h, the number of HH cells stained for annexin V increased from 3% to 63% in a concentration-dependent manner compared to vehicle control. As the incubation time of SAHA at 2.5 μM increased from 24 to 48 h, the number of HH cells stained for annexin V increased from 43% to 63% in a time-dependent manner compared to vehicle control (Fig 1D). Similar results were also seen in the MJ and Hut78 cell lines but again MJ cells were less sensitive to SAHA (Fig 1E). These results were consistent with analysis of cell-cycle distributions from PI staining, further confirming that CTCL cell lines undergo apoptosis following SAHA treatment.

To confirm these findings, we also tested the pro-apoptotic effect of SAHA on freshly isolated PBL from 11 SS/MF patients with high percentage of circulating malignant T-cells and three healthy donors. As the SAHA concentration increased from 1 to 5 μM for 48 h, the number of patients’ PBL stained for annexin V increased from 15% to 32% compared to vehicle control. As the SAHA concentration increased from 1 to 5 μM for 48 h, the number of healthy donors’ PBL stained for annexin V increased from 6% to 13% compared to vehicle control. Thus, patients’ PBL were more sensitive to SAHA treatment than healthy donors’ PBL (p < 0.05) (Table I).

SAHA induced accumulation and modulated apoptosis-associated proteins in CTCL cells To understand the mechanism of SAHA-induced apoptosis, western blotting was used to examine the effects of SAHA on the accumulation of acetylated histones and expression of apoptosis-associated proteins in three CTCL cell lines and patients’ PBL undergoing apoptosis. The acetylated histones (H2B, H3, and H4) were absent or weakly expressed in three CTCL cell lines and patients’ PBL, and increased following SAHA treatment (Fig 2A). The cyclin-dependent kinase (CDK) inhibitor p21/WAF1 was absent in Hut78, HH, and two patients’ PBL but expressed in MJ, and induced following SAHA treatment. The tumor suppressor p53 protein was expressed in MJ, HH, and patients’ PBL but not in Hut78 cells, and did not change following SAHA treatment. Stat3 and Stat6 proteins were expressed and Stat6 but not Stat3 decreased following SAHA treatment. Stat3 and Stat6 proteins were phosphorylated and phospho-Stat6 but not Stat3 decreased following SAHA treatment. SAHA did not change the level of anti-apoptotic protein bcl-2 but increased pro-apoptotic protein bax. Caspase-3 activation and poly (ADP-Ribose) polymerase (PARP) cleavage following SAHA treatment were evident as shown by the appearance of cleaved 20- and 17-, and 85-kDa fragments, respectively (Fig 2B).

Discussion

In CTCL, most effective treatments including phototherapy (Baron and Stevens, 2003), photopheresis (Zic, 2003) and retinoids (Zhang et al., 2002) induce apoptosis of T cells. In this study, we show that SAHA at 1–5 μM selectively causes apoptosis of CTCL cell lines and SS/MF patients’ PBL compared to healthy donors’ PBL. SAHA also induced an accumulation of acetylated histones, an increase of p21/WAF1 and bax, a decrease of Stat6 and phospho-Stat6, and activation of caspase3. Our study agrees with the finding that depsipeptide (FK228), another structural class of HDAC inhibitors, also induces apoptosis of Hut78 CTCL cells in vitro (Piekarz et al., 2004).

An important attribute of HDAC inhibitors including SAHA is that they induce tumor cell apoptosis at concentrations to which normal cells are relatively resistant, making them well suited for cancer therapy (Marks and Jiang, 2005). This difference in sensitivity to SAHA-induced apoptosis appears not to be caused by a difference in the ability to inhibit HDAC activity because accumulation of acetylated histones occurs in both tumor and normal cells (Ungerstedt et al., 2005). In this study, we also showed that SAHA at 1–5 μM selectively causes apoptosis of CTCL cell lines and patients’ PBL compared to healthy donors’ PBL. More recent
studies showed that thioredoxin, a hydrogen donor for many protein targets and a scavenger of ROS, is an important determinant of resistance of cells to SAHA-induced apoptosis (Ungerstedt et al., 2005). In our study, there is also different sensitivity to SAHA in three cell lines and patients’ PBL. Further exploration may be needed to understand the mechanism of sensitivity difference to SAHA in inducing apoptosis of different CTCL cell lines and patients’ PBL.

Chromatin, the basic structural unit of DNA, is composed of nucleosomes with approximately 146 bp of DNA wrapped around an octamer of core histones (two molecules of H2A, H2B, H3, and H4) (Mei et al., 2004; Drummond et al., 2005). Histone acetylation plays an important role in transcriptional regulation. The status of histone acetylation is a dynamic process depending on a balance between the activities of HDAC and histone acetyltransferases (HAT) (Mei et al., 2004; Drummond et al., 2005). HAT acetylate histone lysine substrates, opening up compacted chromatin and promoting binding of transcription factors and gene transcription. In contrast, HDAC decrease acetylation of histone lysine tails, thereby condensing chromatin structure and repressing gene transcription (Mei et al., 2004; Drummond et al., 2005).
Abnormal expression, function or recruitment of HDAC and/or HAT is associated with neoplasia, including lymphoid and myeloid leukemia (Vighushin and Coombes, 2004). In this study we show that SAHA treatment at concentrations causing apoptosis also induces an accumulation of acetylated histones, which may represent a therapeutic marker of drug activity in CTCL patients.

Despite wide distribution of HDAC in chromatin, HDAC inhibitors including SAHA alter the expression of only a small number of genes (2%–17%) by differential display and DNA microarray analysis of gene profiling (Lindemann et al., 2004). Among these genes, the cdk inhibitor p21WAF1 is one of the most commonly induced in several types of transformed cells by SAHA treatment (Richon et al., 2000; Gui et al., 2004). The upregulation of p21WAF1 is responsible for not only cell cycle arrest but also apoptosis induced by HDAC inhibitors (Mei et al., 2004; Somech et al., 2004). In this study, we show that SAHA treatment at concentrations causing apoptosis induced the expression of p21WAF1 protein without cell cycle arrest in all three CTCL cell lines, suggesting that p21WAF1 is involved in SAHA-induced apoptosis of CTCL cells. Furthermore, p21WAF1 is a downstream target of the tumor suppressor p53 and can be regulated in a p53-dependent or -independent manner (Gartel
and Tyner, 1999). To address whether up-regulation of p21WAF1 protein is related to p53, we analyzed CTCL cells for protein expression of p53 following SAHA treatment. Our immunoblot results showed that there was no change of p53. Thus, up-regulation of p21WAF1 by SAHA is likely to be independent of a wild-type p53 status (Vrana et al., 1999; Huang et al., 2000).

HDAC have also been found in association with signal transducers and activators of transcription (Stat) (Rascle et al., 2003). Stat proteins comprise a family of transcription factors latent in the cytoplasm that consists of seven different members: Stat1, 2, 3, 4, 5A, 5B, and 6. After activated by phosphorylation in response to a series of cytokines, growth factors, and hormones, Stat dimerize and translocate to the nucleus where Stat bind to specific DNA promoter sequences and thereby regulate gene expression (Calo et al., 2003). Stat3 is activated by a wide variety of extracellular and intracellular stimuli such as 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). Constitutive activation of Stat3 has been demonstrated to contribute to oncogenesis by stimulating cell proliferation and preventing apoptosis in a large number of solid tumors and hematological malignancies including CTCL (Sommer et al., 2004; Turkson, 2004; Mitchell and John, 2005). In contrast, Stat6 is only primarily activated by IL-4 and IL-13 for the development of Th2 cells (Calo et al., 2003). The activation of Stat6 in malignancies is little known. Constitutive activation of Stat6 has been demonstrated in adult T-cell leukemia/lymphoma (Takemoto et al., 1997), leukemia associated with the p190bcr-abl (Iliaria and Van Etten, 1996), and Hodgkin’s lymphoma (Skinnider et al., 2002). In addition, human lymphocytes with Stat6 null phenotype exhibit increased apoptosis (Galka et al., 2004). In this study, we found for the first time that SAHA at concentrations causing apoptosis decreases the expression of Stat6 and phospho-Stat6 but not Stat3 and phospho-Stat3 in three cell lines and patients’ PBL. Thus, down-regulation of Stat6 and phospho-Stat6 may be involved in SAHA-induced apoptosis of CTCL cells.

The balance between expression of anti-apoptotic protein bcl-2 and pro-apoptotic protein bax is critical in controlling the activation of caspases by regulating release of cytochrome c from mitochondria (Manion and Hockenbery, 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). Among the 16 known members of interleukin-1-converting enzyme family of proteases, caspase-3 has been shown to be a key component of the apoptotic machinery (Porter and Janicke, 1999). Caspase-3 is activated in apoptotic cells and cleaves several cellular proteins, including PARP. The cleavage of PARP is a hallmark of apoptosis by various antitumor agents (Duriez and Shah, 1997). We have previously reported that caspase-3 is activated and PARP is cleaved in apoptotic CTCL cells induced by bexarotene (Zhang et al., 2002).

Table I. SAHA selectively induced apoptosis of SS/MF patients’ PBL

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age/gender</th>
<th>Diagnosis</th>
<th>CD4 + CD26− (%)</th>
<th>1</th>
<th>2.5</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>75/F</td>
<td>SS</td>
<td>95</td>
<td>19</td>
<td>34</td>
<td>45</td>
</tr>
<tr>
<td>2</td>
<td>69/F</td>
<td>SS</td>
<td>82</td>
<td>35</td>
<td>58</td>
<td>59</td>
</tr>
<tr>
<td>3</td>
<td>61/F</td>
<td>SS</td>
<td>83</td>
<td>8</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>4</td>
<td>44/F</td>
<td>MF</td>
<td>40</td>
<td>3</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>76/F</td>
<td>SS</td>
<td>91</td>
<td>11</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>6</td>
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<td>67</td>
<td>35</td>
<td>55</td>
<td>56</td>
</tr>
<tr>
<td>8</td>
<td>81/F</td>
<td>MF</td>
<td>37</td>
<td>12</td>
<td>23</td>
<td>20</td>
</tr>
<tr>
<td>9</td>
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<td>61</td>
<td>4</td>
<td>21</td>
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<tr>
<td>10</td>
<td>59/F</td>
<td>SS</td>
<td>79</td>
<td>9</td>
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</tr>
<tr>
<td>11</td>
<td>54/M</td>
<td>SS</td>
<td>97</td>
<td>6</td>
<td>16</td>
<td>18</td>
</tr>
</tbody>
</table>

Statistic analysis

Total (mean ± SD)

<table>
<thead>
<tr>
<th></th>
<th>Patients (n=11)</th>
<th>Healthy donors (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptosis (%) (48 h)a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentrations (µM)</td>
<td>15 ± 11</td>
<td>6 ± 2</td>
</tr>
<tr>
<td></td>
<td>29 ± 17</td>
<td>11 ± 3</td>
</tr>
<tr>
<td></td>
<td>32 ± 18</td>
<td>13 ± 5</td>
</tr>
<tr>
<td>p value</td>
<td>p = 0.03</td>
<td>p = 0.006</td>
</tr>
<tr>
<td></td>
<td>p = 0.009</td>
<td></td>
</tr>
</tbody>
</table>

aApoptosis was determined by flow cytometry analysis of annexin V/PI staining. The values represented the percentage of annexin V + PI− and annexin V + PI− binding from PBL treated with different concentrations of SAHA over vehicle control.

SAHA, suberoylanilide hydroxamic acid; SS, Sézary syndrome; MF, mycosis fungoides; PBL, peripheral blood lymphocytes; M, male; F, female.
and CDDO (Zhang et al., 2004). In this study, we show that SAHA at concentrations causing apoptosis did not change bcl-2 but increased bax, activated caspase-3, and cleaved PARP in three CTCL lines and patients’ PBL. Thus, alteration of bax/bcl-2 ratio, activation of caspase-3, and cleavage of PARP are likely involved in SAHA-induced apoptosis of CTCL cells.

In conclusion, we have demonstrated that SAHA selectively induces apoptosis of CTCL cell lines and freshly isolated PBL from SS/MF patients with high percentage of circulating malignant T-cells. These events are associated with an accumulation of acetylated histones, an increase of p21WAF1, an alteration of bax/bcl-2 ratio, a decrease of Stat6 and phospho-Stat6 proteins, an activation of caspase-3, and a cleavage of PARP. These findings support the therapeutic action of SAHA in ongoing phase I and II clinical trials for CTCL patients. Further exploration may be needed to identify more targeted genes of SAHA that could be used as surrogate end points to analyze SAHA and other HDAC inhibitors in clinical development.

Materials and Methods

Reagents SAHA was provided by Merck (Boston, Massachusetts). SAHA was dissolved in DMSO to stock concentration of 10 mM and stored at −20°C. Serial dilutions (1, 2.5, and 5 μM) were freshly made in RPMI 1640.

Cells and cell culture Human CTCL cell lines MJ (G11), Hut78, and HH obtained from American Type Culture Collection (Rockville, Maryland) were derived from peripheral blood of patients with MF, SS, and non-MF/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 eleven SS/MF patients with high percentage of...
circuitulating malignant T cells (Table I). Samples were obtained during routine diagnostic assessments after informed consent was obtained in accordance with regulations and protocols sanctioned by the Human Subjects Committee of M. D. Anderson. The study was conducted according to the Declaration of Helsinki Principles. PBL from these patients and healthy donors were isolated by Vacutainer CPT (Becton Dickinson, San Jose, California). Cells were grown in RPMI 1640 medium (Sigma Chemical, St. Louis, Missouri) supplemented with 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, Utah), 2 mM HEPES, and 1% penicillin-streptomycin in a humidified atmosphere with 5% CO₂ at 37°C.

Cell viability Cell viability was measured by CellTiter 96 AQ One Solution Cell Proliferation Assay (Goodwin et al, 1995) according to the manufacturer’s instructions (Promega, Madison, Wisconsin). Aliquots of 5 × 10⁵ cells per well were distributed in 96-well microplates (Falcon; New Jersey) in 100 μL of medium and incubated with SAHA (1, 2.5, and 5 μM) for 24 and 48 h, 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 h. 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.

Flow cytometry analysis of cell cycle MJ, Hut78, and HH4 cells (1 × 10⁶ cells) were incubated with or without SAHA (1, 2.5, 5 μM) for 24 and 48 h. Cells were collected, washed with cold phosphate-buffered saline (PBS), fixed in cold (−20°C) 100% ethanol, treated with DNase-free RNase, and stained with 50 μg per mL propidium iodide (PI). Distribution of the cell-cycle phase by different DNA content was determined with a FACScan flow cytometer (Becton Dickinson). For each sample, 10,000-gated events were acquired. Analyses of cell-cycle distribution (including apoptosis: sub-G₁) were performed using Modfit software (Becton Dickinson).

Annexin V binding staining The analysis of annexin V binding was carried out with an Annexin V-FITC Detection Kit I (PharMingen, San Diego, California) according to the manufacturer’s instructions. Briefly, three cell lines and PBL from healthy donors and patients were incubated with or without SAHA (1, 2.5, and 5 μM) for 24 and/or 48 h. Cells were collected, washed twice with cold PBS, and centrifuged at 500 × g for 5 min. Cells were resuspended in 1 × binding buffer at a concentration of 1 × 10⁶ cells per mL, 100 μL of the solution were transferred to a 5 mL culture tube, and 5 μL of annexin V-FITC and 5 μL of PI were added. Cells were gently vortexed and incubated for 15 min at room temperature in the dark. Finally, 400 μL of 1 × binding buffer was added to each tube, and samples were analyzed by FACSscan flow cytometer (Becton Dickinson). For each sample, 10,000 ungated events were acquired. Annexin V−PI− cells represent the early apoptotic populations. Annexin V−PI+ cells represent either late apoptotic or secondary necrotic populations.

Isolation of cytoplasmic and nuclear extracts Cells (5 × 10⁶) were washed with ice-cold PBS, harvested into 1 mL of PBS, 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, 1 mM DTT, and 1 × protease inhibitor cocktail “complete mini” (Roche, Indianapolis, Indiana)). After a 20-min incubation on ice, the mixture was treated with a 24-G syringe for five times 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 DTT, 10% glycerol, and 1 × protease inhibitor cocktail “complete mini” (Roche)) and incubated at 4°C with shaking for 15 min. Protein concentrations were determined by the Bradford dye-binding protein assay (Bio-Rad, Richmond, California) using bovine serum albumin as a standard.

Western blot analysis Western blot analysis was carried out as described previously (Zhang et al, 2002). Briefly, cytoplasmic (10 μg) or nuclear (5 μg) proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel (8%–12%) electrophoresis (SDS-PAGE) 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), incubated with primary antibody overnight at 4°C in 3% powdered milk in TBST, and washed extensively with TBST. They were incubated with 1:5000 peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, Pennsylvania) for 1 h at room temperature. Polyclonal rabbit anti-acetyl-histones (H2B, H3 and H4), and anti-Stat6 and bax antibodies were obtained from Upstate Biotechnology (Lake Placid, New York) and Santa Cruz Biotechnology (Santa Cruz, California), respectively. Monoclonal mouse anti-p21MAPK and anti-Stat3 antibodies were obtained from Oncogene (Boston, Massachusetts) and BD Biosciences (San Diego, California), respectively. Monoclonal mouse anti-p53, bcl-2, pro-caspase-3, and PARP antibodies were obtained from Santa Cruz Biotechnology. Monoclonal mouse anti-phospho-Stat3, phospho-Stat6, and rabbit anti-caspase-3 antibodies were obtained from Cell Signaling (Beverly, Massachusetts). Immunoreactive bands were visualized by enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, UK). The equivalent loading of proteins in each well was confirmed by actin and Ponceau staining.

Statistical analysis All experiments were performed in triplicate unless otherwise noted. Results were expressed as means ± SD. Statistical significance was evaluated by Student’s t test, and p < 0.05 was considered as significant.

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