

# Curcumin Selectively Induces Apoptosis in Cutaneous T-Cell Lymphoma Cell Lines and Patients' PBMCs: Potential Role for STAT-3 and NF- $\kappa$ B Signaling

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Curcumin inhibits cell growth and induces apoptosis in a number of tumor cell lines and animal models. Human clinical trials indicated no dose-limiting toxicity when administered at doses up to 8 g per day. The purpose of this study was to address the antitumor effect of curcumin on cutaneous T-cell lymphoma (CTCL) cell lines and peripheral blood mononuclear cells (PBMCs) from patients with CTCL compared with healthy donors' controls. Curcumin at 5–20  $\mu$ M for 24 and 48 hours induced apoptosis in a time- and dose-dependent manner in three CTCL cell lines (namely MJ, Hut78, and HH). Curcumin at 5–20  $\mu$ M for 48 hours also caused more apoptosis in patients' PBMCs compared with healthy donors' PBMCs ( $P < 0.05$ ). Curcumin decreased protein and mRNA expression levels of signal transducer and activator of transcription (STAT)-3, bcl-2, and survivin in three cell lines and in patients' PBMCs. Curcumin inhibited STAT-3 and I $\kappa$ B- $\alpha$  phosphorylation, as well as suppressed DNA binding of nuclear factor (NF)- $\kappa$ B in these cells. Caspase-3 was activated and poly (ADP-Ribose) polymerase was cleaved after curcumin treatment. These data suggest that curcumin selectively induces apoptosis in association with the downregulation of STAT-3 and NF- $\kappa$ B signaling pathways in CTCL cells. Our findings provide a mechanistic rationale for the potential use of curcumin as a therapeutic agent for patients with CTCL.

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## INTRODUCTION

Cutaneous T-cell lymphomas (CTCLs) are extranodal non-Hodgkin's lymphomas with pleomorphic skin lesions and distinct T-cell markers. The incidence of CTCL at 6.4 per million has doubled since the last analysis and it remains more common in males and in African Americans (Criscione and Weinstock, 2007). Mycosis fungoides (MF), the most common and indolent form of CTCL, is characterized by epidermotropic CD4 + CD45RO + CLA + CCR + helper/memory T cells, which may lack CD7 and/or CD26 expression. Sézary syndrome (SS), a leukemic variant, is characterized with erythroderma and atypical cells in the peripheral blood and by a peripheral blood T-cell clone (Duvic and Foss, 2007). These malignant T cells exhibit abnormal apoptotic mechanisms, such as loss of Fas or

expression of bcl-2, which result in the loss of activation-induced cell death, prolonged life span, and accumulation (Kacinski and Flick, 2001). There are a limited number of Food and Drug Administration-approved therapeutic modalities available to treat patients with MF/SS, including bexarotene, intravenous denileukin diftitox, photopheresis, and vorinostat (Duvic *et al.*, 2001, 2007; Duvic and Foss, 2007). However, a number of nonapproved agents are commonly used as a standard of care and include topical steroids and mustargen, phototherapy, interferons, and chemotherapy. Patients with refractory or transformed MF/SS have a poor prognosis and therapy is usually palliative (Kim *et al.*, 2003). Alternative or complementary therapies, especially for advanced MF/SS, are required.

Curcumin (diferuloylmethane) is the active compound in turmeric (*Curcuma longa*), a dietary spice that has been widely consumed for many centuries (Ammon and Wahl, 1991). This compound has been shown to interact with a wide variety of proteins modifying their expression and activity (Kunnumakkara *et al.*, 2008). These include inflammatory cytokines and enzymes, transcription factors, and gene products linked to cell survival, proliferation, invasion, and angiogenesis. Curcumin has been found to exert antiproliferative and proapoptotic effects in a number of tumor cell lines *in vitro* (Moragoda *et al.*, 2001; Bharti *et al.*, 2003; Li *et al.*, 2004; Odot *et al.*, 2004; Uddin *et al.*, 2005). In murine carcinoma models, curcumin has also been shown to have chemopreventive effects, to reduce the number and size

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Abbreviations: CTCL, cutaneous T-cell lymphoma; CQ, chloroquine; MF, mycosis fungoides; NF- $\kappa$ B, nuclear factor- $\kappa$ B; PARP, poly (ADP-Ribose) polymerase; PBMC, peripheral blood mononuclear cell; PI, propidium iodide; SS, Sézary syndrome; STAT, signal transducer and activator of transcription  
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of tumors, and in some cases, to even ameliorate tumor-induced immunodepletion of the host (Huang *et al.*, 1988, 1994; Tanaka *et al.*, 1994; Pal *et al.*, 2001).

Phase I studies of curcumin have shown that this agent can be administered safely at oral doses of up to 8 g per day with no dose-limiting toxicity (Cheng *et al.*, 2001; Sharma *et al.*, 2001, 2004). More recently, a phase II study of curcumin has shown that this compound has biological activity in some patients with pancreatic cancer, although its usefulness may be attenuated because of its poor oral bioavailability (Dhillon *et al.*, 2008). Various approaches are being pursued to overcome these limitations, which include synthesis of curcumin analogs, the use of adjuvants (such as piperine), and the development of improved delivery platforms for the parental compound, such as liposomal, nanoparticulated, and phospholipid complex formulations of curcumin (Anand *et al.*, 2007). Furthermore, this compound has been shown to have therapeutic effects in other skin diseases, such as psoriasis, scleroderma, and malignant melanoma (Heng *et al.*, 2000; Odot *et al.*, 2004; Tourkina *et al.*, 2004). In this study, we examined the antitumor effects of curcumin on CTCL cell lines and peripheral blood mononuclear cells (PBMCs) from MF/SS patients with a high percentage of circulating tumor T cells compared with healthy donors' controls.

## RESULTS

### Curcumin treatment inhibited cell growth in CTCL cell lines

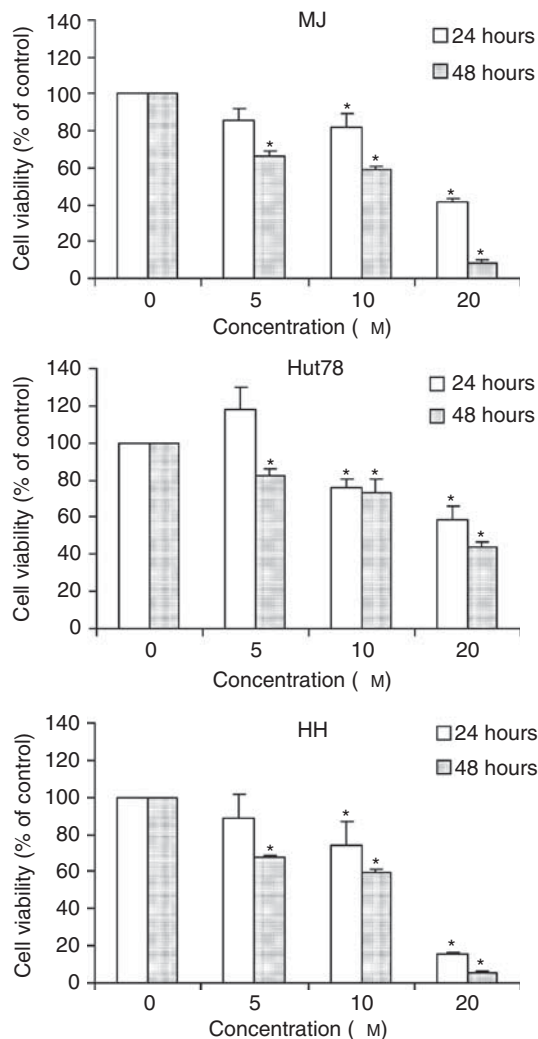
To determine whether curcumin can inhibit CTCL cell growth, MJ, Hut78, and HH cells were treated with or without 5, 10, and 20  $\mu\text{M}$  curcumin for 24 and 48 hours, and their viability was evaluated by CellTiter 96 Aqueous One Solution Cell Proliferation Assay. As the concentrations of curcumin increased from 5 to 20  $\mu\text{M}$  over 24 and 48 hours, the cell growth of HH cells was inhibited by 11.7–84.9% and 32.8–94.9% in a dose- and a time-dependent manner, respectively, compared with vehicle control ( $n=3$ ;  $P<0.05$ ). Similar results were also seen in MJ and Hut78 cell lines (Figure 1). These results show that curcumin is an inhibitor of CTCL cell line growth *in vitro*.

### Curcumin treatment induced apoptosis in CTCL cell lines

To determine whether the growth inhibition of CTCL cells by curcumin results from apoptosis, MJ, Hut78, and HH cell lines were treated with or without 5, 10, and 20  $\mu\text{M}$  curcumin for 24 and 48 hours. As curcumin concentrations increased from 5 to 20  $\mu\text{M}$  for 24 and 48 hours, the number of HH cells staining for annexin V increased by 2.7–36.3% and 4.7–71.7% in a dose- and time-dependent manner, respectively, compared with vehicle control ( $n=3$ ;  $P<0.05$ ). Similar results were also seen in MJ and Hut78 cell lines; however, sensitivity to curcumin is different among the three CTCL cell lines (Figure 2).

### Curcumin treatment selectively induced apoptosis of MF/SS patients' PBMCs

To confirm the results from cell lines, we also tested the proapoptotic effect of curcumin on freshly isolated PBMCs from eight MF/SS patients with a high percentage

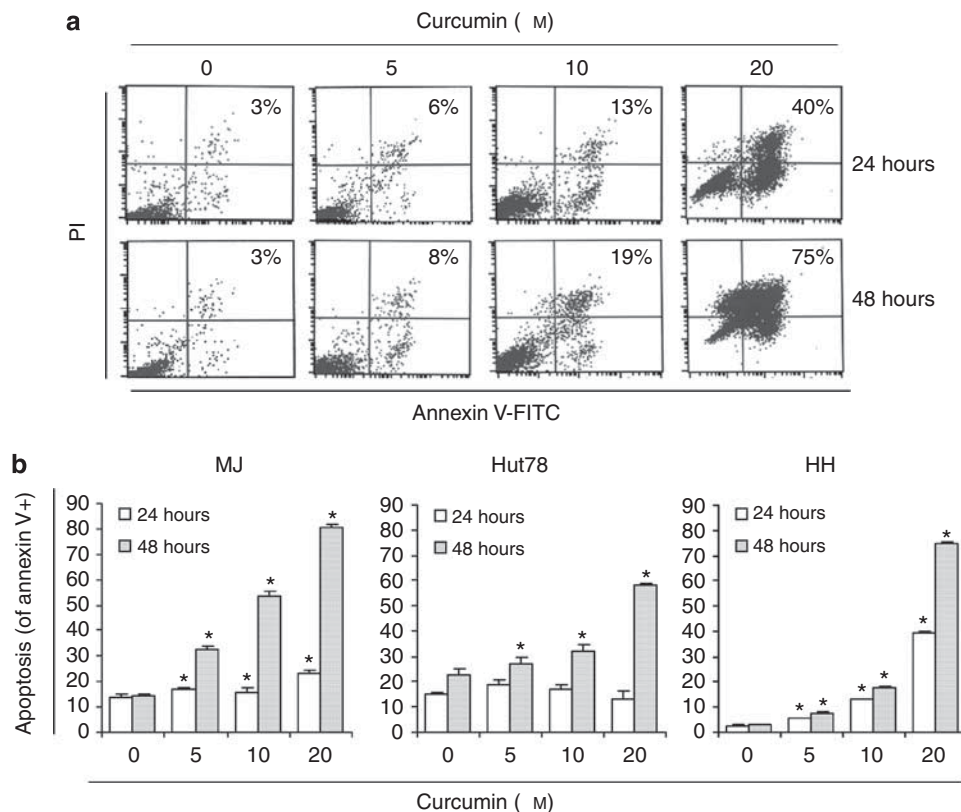


**Figure 1. Effect of curcumin on cell viability in CTCL cell lines.** Cells ( $5 \times 10^4$ ) were aliquoted into a 96-well culture plate and grown in medium with or without 5, 10, or 20  $\mu\text{M}$  curcumin for 24 or 48 hours. Cell viability was determined by comparing untreated controls with treated groups using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay performed in triplicate. Each point represents mean  $\pm$  SD of triplicate determinations. \*Significantly different from control values ( $n=3$ ;  $P<0.05$ ). CTCL, cutaneous T-cell lymphoma.

(43.8–98.2%) of circulating  $\text{CD4}^+\text{CD26}^-$  tumor T cells and from three normal donors. As curcumin concentrations increased from 5 to 20  $\mu\text{M}$  for 48 hours, the number of patients' PBMCs stained for annexin V increased by 9.1–59.2% compared with vehicle control. As curcumin concentrations increased from 5 to 20  $\mu\text{M}$  for 48 hours, the number of healthy donors' PBMCs stained for annexin V increased by 2.4–24.6% compared with vehicle control. Thus, curcumin induced more apoptosis in MF/SS patients' PBMCs than healthy donors' PBMCs ( $P<0.05$ ) (Table 1).

### Curcumin treatment decreased expression of STAT-3 and STAT-3-regulated antiapoptotic proteins in CTCL cell lines and in patients' PBMCs

Constitutive activation of signal transducer and activator of transcription (STAT)-3 signaling has been shown to contribute



**Figure 2. Effect of curcumin on apoptosis induction in CTCL cell lines.** Three cell lines were treated with or without 5, 10, or 20  $\mu\text{M}$  curcumin for 24 or 48 hours. Annexin V binding was carried out using the Annexin V-FITC Detection Kit. (a) Curcumin induced a concentration- and time-dependent apoptosis in the HH cell line. (b) Each point represents the percentage (mean  $\pm$  SD of triplicate determinations) of both annexin V<sup>+</sup>PI<sup>-</sup> and annexin V<sup>+</sup>PI<sup>+</sup> cells. \*Significantly different from control values ( $n=3$ ;  $P<0.05$ ). CTCL, cutaneous T-cell lymphoma; FITC, fluorescein isothiocyanate; PI, propidium iodide.

to oncogenesis by stimulating cell proliferation and preventing apoptosis in CTCL (Eriksen *et al.*, 2001; Nielsen *et al.*, 2002; Sommer *et al.*, 2004). To determine whether STAT signaling is involved in curcumin-induced apoptosis, we examined the expression of STAT-3 and p-STAT-3 and their regulated antiapoptotic proteins (bcl-2 and survivin) in three CTCL cell lines (namely MJ, Hut78, and HH) and in four MF/SS patients' PBMCs after curcumin treatment at 10  $\mu\text{M}$  for 24 hours. Western blotting results showed that expression levels of both STAT-3 and p-STAT-3 proteins decreased by a range of 21–55% and 38–47%, respectively, in these CTCL cells compared with untreated controls (Figure 3a). Moreover, quantitative real-time PCR results showed that mRNA expression levels of STAT-3 also decreased by 23–57% compared with untreated controls (Figure 3b). Similar to STAT-3, protein and mRNA expression levels of bcl-2 decreased by 22–94% and by 54–92%, respectively; and protein and mRNA expression levels of survivin decreased by 11–95% and 50–83%, respectively compared with untreated controls (Figure 4).

#### Curcumin treatment inhibited DNA binding of NF- $\kappa$ B and I $\kappa$ B- $\alpha$ phosphorylation in CTCL cell lines and in patients' PBMCs

The transcriptional factor nuclear factor (NF)- $\kappa$ B is constitutively activated in CTCL and has been implicated in the

pathogenesis of this disorder (Sors *et al.*, 2006; Döbbling, 2007). To figure out whether NF- $\kappa$ B activation is involved in curcumin-induced apoptosis, we examined DNA binding of NF- $\kappa$ B in three CTCL cell lines (MJ, Hut78, and HH) and in two patients' PBMCs by electrophoretic mobility-shift assay. Our results show that curcumin treatment at 10  $\mu\text{M}$  for 24 hours inhibited the DNA binding of NF- $\kappa$ B in CTCL cell lines and in patients' PBMCs (Figure 5a). Moreover, to investigate whether the inhibitory effect of curcumin is mediated through the alteration of phosphorylation of I $\kappa$ B- $\alpha$ , these CTCL cells were treated with curcumin, and their protein extracts were checked for p-I $\kappa$ B- $\alpha$  expression. The results showed that untreated CTCL cells constitutively expressed p-I $\kappa$ B- $\alpha$ . Upon curcumin treatment, the ratio of p-I $\kappa$ B- $\alpha$  and I $\kappa$ B- $\alpha$  decreased by 25–56% compared with untreated controls (Figure 5b).

#### Curcumin treatment activated caspase-3 and cleaved PARP in CTCL cell lines and in patients' PBMCs

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 curcumin-induced apoptosis, we examined the expression of caspase-3 and poly (ADP-Ribose) polymerase (PARP) proteins in three CTCL cell lines (MJ, Hut78, and HH) and in four

**Table 1. Curcumin selectively induced apoptosis of MF/SS patients' PBMCs**

| Patient no.        | Age/gender                     | Diagnosis | CD4+CD26- (%) | Apoptosis % <sup>1</sup> (48 h) |             |             |
|--------------------|--------------------------------|-----------|---------------|---------------------------------|-------------|-------------|
|                    |                                |           |               | Concentrations (μM)             |             |             |
|                    |                                |           |               | 5                               | 10          | 20          |
| 1                  | 55/F                           | MF/SS     | 46.3          | 15.8%                           | 54.9%       | 80.2%       |
| 2                  | 61/F                           | MF        | 43.8          | 0.5%                            | 22.9%       | 68.8%       |
| 3                  | 70/M                           | SS        | 95.8          | 8.9%                            | 14.4%       | 71.4%       |
| 4                  | 53/F                           | MF/SS     | 98.2          | 2.8%                            | 8.0%        | 49.8%       |
| 5                  | 58/M                           | MF/SS     | 96.4          | 15.0%                           | 23.5%       | 42.7%       |
| 6                  | 51/M                           | SS        | 77.1          | 13.7%                           | 29.4%       | 46.2%       |
| 7                  | 67/F                           | SS        | 87.0          | 3.2%                            | 19.4%       | 44.0%       |
| 8                  | 71/M                           | SS        | 93.0          | 13.2%                           | 40.3%       | 70.7%       |
| Statistic analysis |                                |           |               |                                 |             |             |
| Total (mean±SD)    | Patients (n=8)                 |           |               | 9.1 ± 6.2                       | 26.6 ± 15.0 | 59.2 ± 15.0 |
|                    | Healthy donors (n=3)           |           |               | 2.4 ± 6.3                       | 11.6 ± 5.8  | 24.6 ± 5.5  |
| P-value            | Patients versus healthy donors |           |               | P=0.20                          | P=0.04      | P=0.0004    |

Abbreviations: MF, mycosis fungoides; PBMC, peripheral blood mononuclear cell; PI, propidium iodide; SS, Sézary syndrome.

<sup>1</sup>Apoptosis was determined by flow cytometry analysis of annexin V/PI staining. The values represented the percentage of annexin V<sup>+</sup>PI<sup>-</sup> and annexin V<sup>+</sup>PI<sup>+</sup> binding from PBMCs treated with different concentrations of curcumin over vehicle control.

patients' PBMCs. Western blotting results showed that curcumin treatment activated caspase-3 and cleaved PARP as determined by the appearance of cleaved 17- and 20-kDa, as well as 82-kDa fragments in CTCL cell lines and in patients' PBMCs, respectively (Figure 4a).

#### The autophagy inhibitor protected curcumin-induced cell growth inhibition in CTCL cell lines

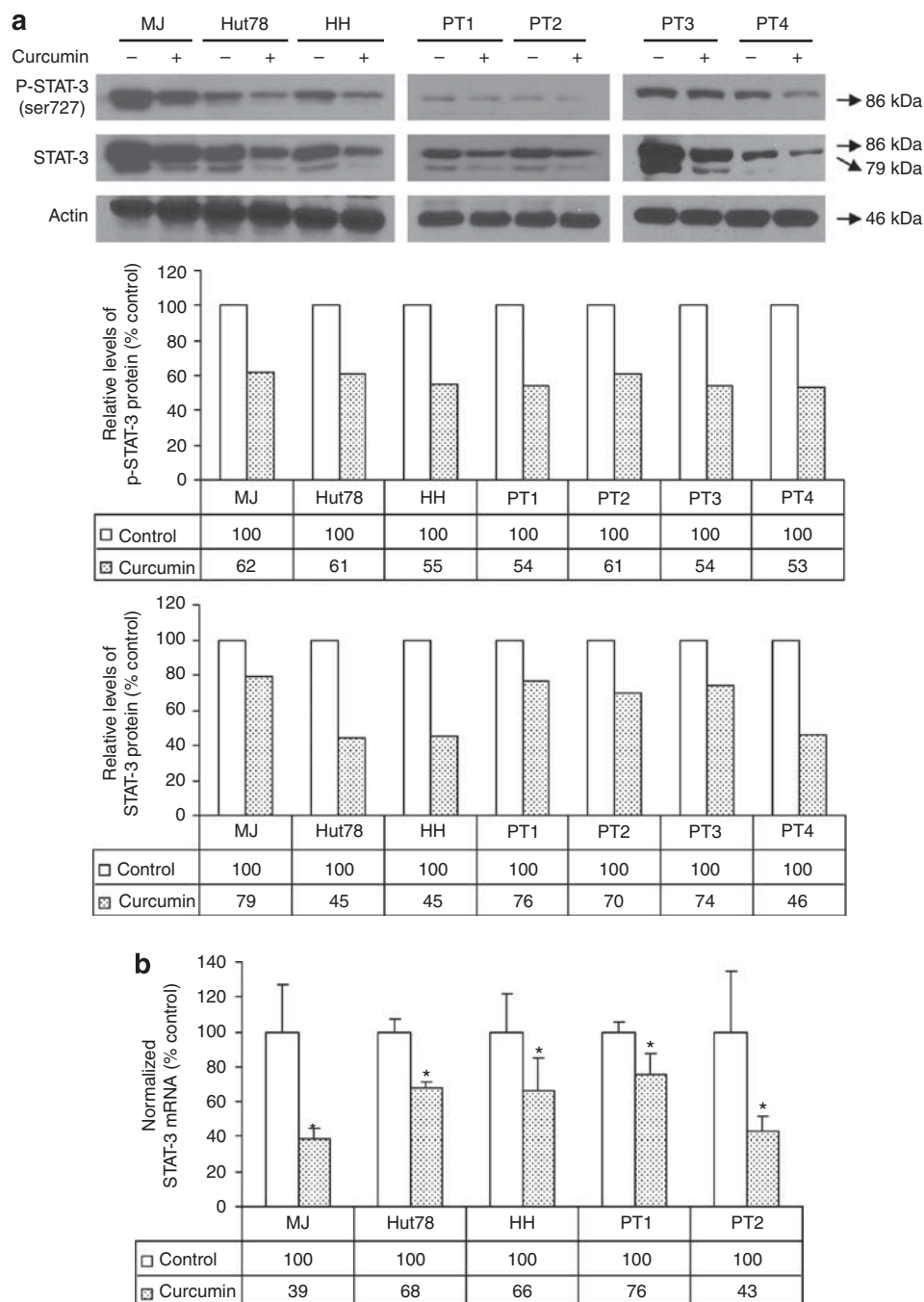
Although apoptosis induced by curcumin was associated with caspase-3 activation in CTCL cells, we cannot exclude other types of caspase-3-independent cell death. Thus, we hypothesized that curcumin may also induce autophagy. Three CTCL cell lines (MJ, Hut78, and HH) were pretreated with 10 μM chloroquine (CQ), an inhibitor of autophagy (Zhang et al., 2008), for 24 hours and then incubated with curcumin at 10 μM for 48 hours. Our results showed that three CTCL cell lines pretreated with CQ improved cell viability relative to cells treated with curcumin alone by 12–19% (Figure 6).

#### DISCUSSION

As defective apoptosis of skin-homing memory T cells is involved in the pathogenesis of CTCL, pharmacological modulation of apoptosis may provide new therapeutic approaches (Kacinski and Flick, 2001; Meech et al., 2001). The most effective treatments for MF/SS, including phototherapy (Baron and Stevens, 2003), photopheresis (Zic, 2003), the RXR (retinoid x receptor) selective retinoid bexarotene (Zhang et al., 2002), and the histone deacetylase

inhibitor vorinostat (Zhang et al., 2005), induce apoptosis of tumor T cells. In this study, we report that curcumin at 5–20 μM caused apoptosis in CTCL cell lines. More importantly, our results showed that curcumin treatment preferentially induced more apoptosis in PBMCs from patients with a high percentage of circulating tumor T cells compared with healthy donor controls. Previous studies have shown that curcumin can selectively kill transformed tumor cells from colon, breast, and liver cancers compared with normal cells *in vitro* (Jiang et al., 1996; Ramachandran and You, 1999; Choudhuri et al., 2005; Watson et al., 2008). Moreover, curcumin selectively induced apoptosis in chronic lymphocytic leukemia patients' primary cells compared with healthy donors' mononuclear cells (Everett et al., 2007). The selectivity of curcumin for cancer cells might be explained by its ability to increase the cellular levels of reactive oxygen species (Lopez-Lazaro, 2008). In addition, there is also different sensitivity to curcumin in the three cell lines and patients' PBMCs. Further exploration is required to understand the molecular basis of sensitivity difference to curcumin in inducing apoptosis of different CTCL cell lines and patients' cells.

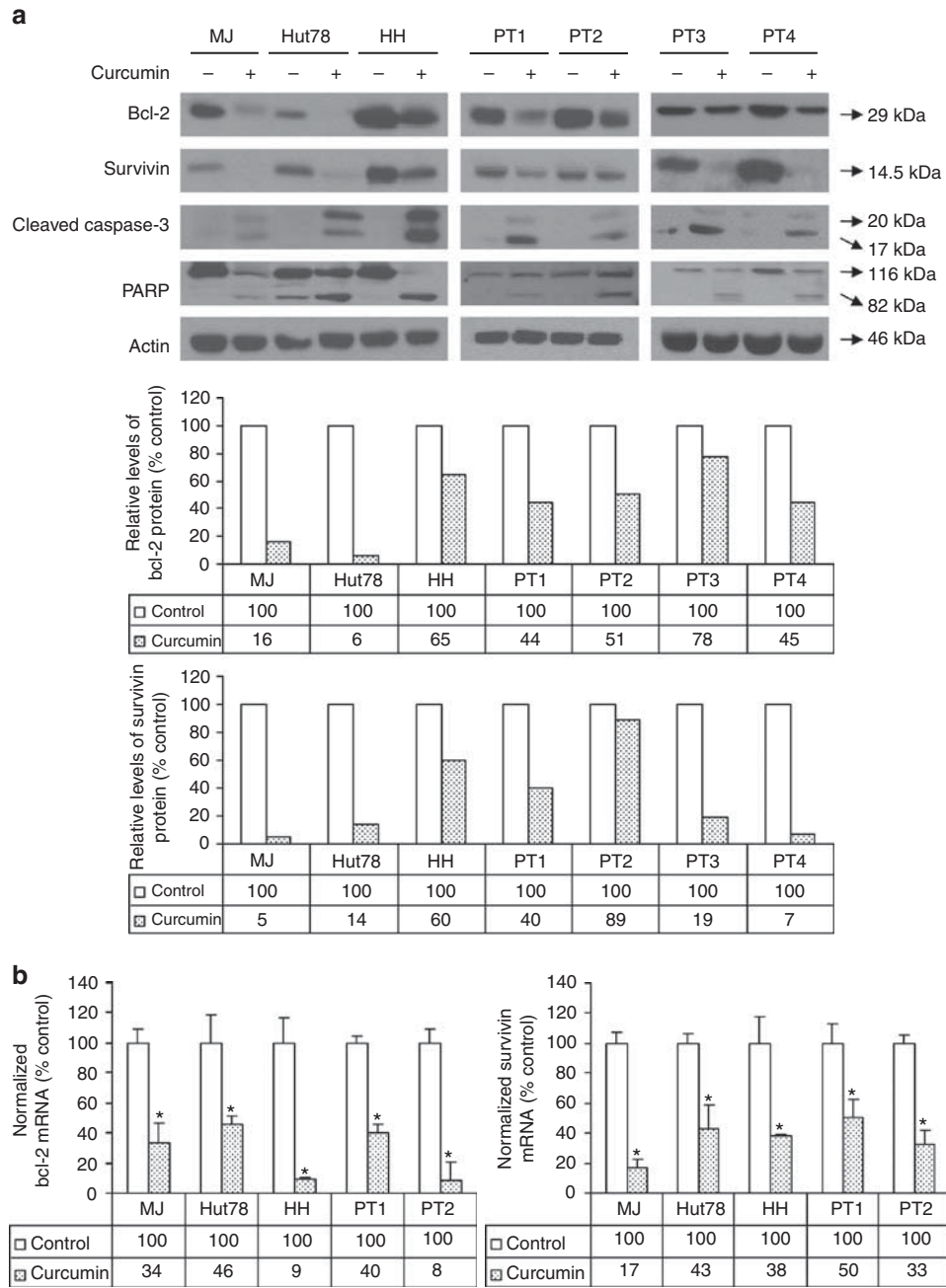
A possible mechanism underlying the induction of apoptosis by curcumin could be its capacity to inhibit NF-κB and STAT-3 signaling. NF-κB is well known as a transcription factor that regulates the expression of a large number of genes in response to various cellular conditions, including infection, inflammation, adhesion, cell cycle, and survival. Activation of NF-κB can be controlled through the alteration



**Figure 3. Effect of curcumin on the expression of STAT-3/p-STAT-3 proteins and STAT-3 mRNA in CTCL cell lines and in patients' PBMCs.** CTCL cells were treated with or without 10  $\mu$ M curcumin for 24 hours. (a) Expression of STAT-3 and p-STAT-3 proteins was evaluated by western blots. The protein levels were quantified and expression levels of STAT-3 protein from untreated control cells were used as 100%. (b) Expression of STAT-3 mRNA was examined by quantitative real-time PCR assays. The expression levels of normalized STAT-3 mRNA from untreated control cells were used as 100%. The results were expressed as mean  $\pm$  SD in three independent experiments. \*Significantly different from control values ( $n=3$ ;  $P<0.05$ ). CTCL, cutaneous T-cell lymphoma; PBMC, peripheral blood mononuclear cell; STAT, signal transducer and activator of transcription.

of phosphorylation of the I $\kappa$ B inhibitory protein, such as I $\kappa$ B- $\alpha$  by I $\kappa$ B- $\alpha$  kinase (Aggarwal, 2004). In CTCL, constitutive nuclear NF- $\kappa$ B activity is required for cell survival and resistance to apoptosis in CTCL cells (Sors *et al.*, 2006; Döbbeling, 2007). This is manifested by the phosphorylation of STAT-3, which results in impaired tumor cell apoptosis

(Eriksen *et al.*, 2001; Nielsen *et al.*, 2002; Sommer *et al.*, 2004). Constitutive activation of STAT-3 has also been shown 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). Agents

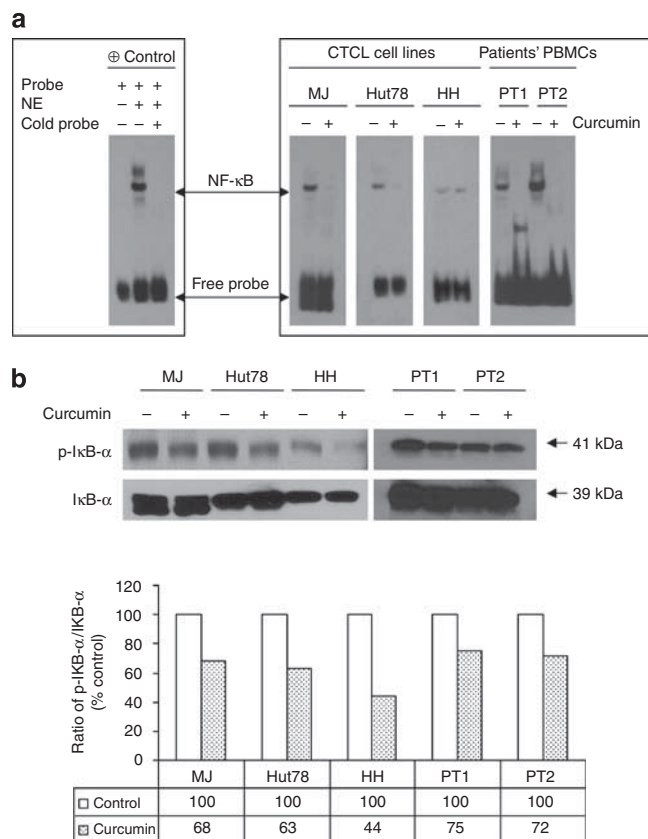


**Figure 4. Effect of curcumin on the expression of apoptosis-related proteins and mRNAs in CTCL cell lines and in patients' PBMCs.** CTCL cells were treated with or without 10  $\mu$ M curcumin for 24 hours. (a) Expression of apoptosis-related proteins was evaluated by western blots. The protein levels were quantified and expression levels of bcl-2 and survivin proteins from untreated control cells were used as 100%. (b) Expression of bcl-2 and survivin mRNAs was examined by quantitative real-time PCR assay. The expression levels of normalized bcl-2 and survivin mRNAs from untreated control cells were used as 100%. The results were expressed as mean  $\pm$  SD in three independent experiments. \*Significantly different from control values ( $n=3$ ;  $P<0.05$ ). CTCL, cutaneous T-cell lymphoma; PBMC, peripheral blood mononuclear cell.

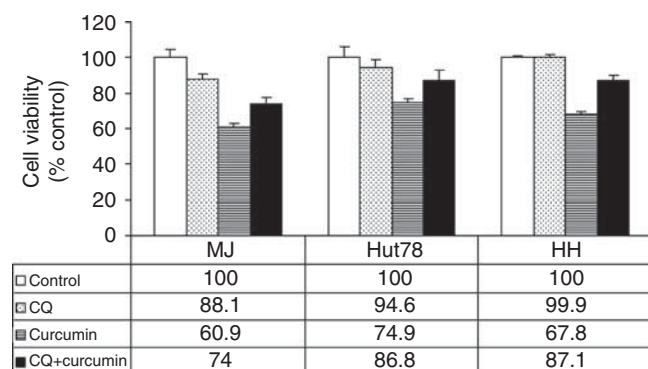
that suppress NF- $\kappa$ B and STAT-3 activity could be good candidates for the treatment of CTCL, as they should be able to induce apoptosis in CTCL cells. In this study, curcumin treatment at concentrations causing apoptosis decreased the protein and mRNA expression of STAT-3, as well as inhibited activation of STAT-3 in CTCL cells. Curcumin treatment also inhibited DNA binding of NF- $\kappa$ B through the suppression of

I $\kappa$ B- $\alpha$  phosphorylation in CTCL cells. Thus, downregulation of STAT-3 signaling and inhibition of NF- $\kappa$ B activity may be involved in curcumin-induced apoptosis of CTCL cells.

CTCL tumors remain restricted to the skin for most of the time as the disease progresses. The low number of mitotic cells led to the idea that the increase in tumor mass is due to the overexpression of cell survival genes such as bcl-2, which



**Figure 5. Curcumin inhibited DNA binding of NF- $\kappa$ B and I $\kappa$ B- $\alpha$  phosphorylation in CTCL cell lines and in patients' PBMCs.** CTCL cells were treated with or without 10  $\mu$ M curcumin for 24 hours. (a) DNA-binding activity of NF- $\kappa$ B was determined by EMSA using biotin-labeled NF- $\kappa$ B probe. (b) Expression of p-I $\kappa$ B- $\alpha$  and I $\kappa$ B- $\alpha$  proteins was evaluated by western blots. The protein levels were quantified and the ratio of p-I $\kappa$ B- $\alpha$  and I $\kappa$ B- $\alpha$  protein expression from untreated control cells was used as 100%. CTCL, cutaneous T-cell lymphoma; EMSA, electrophoretic mobility-shift assay; NF- $\kappa$ B, nuclear factor- $\kappa$ B; PBMC, peripheral blood mononuclear cell.



**Figure 6. The autophagy inhibitor protected curcumin-induced cell growth inhibition in CTCL cell lines.** Three CTCL cell lines were pretreated with or without 10  $\mu$ M chloroquine (CQ) for 24 hours, followed by 10  $\mu$ M curcumin for 48 hours. Cell viability was measured using the CellTiter 96 AQueous One Solution Cell Proliferation Assay performed in triplicate. Each point represented mean  $\pm$  SD of triplicate determinations. CTCL, cutaneous T-cell lymphoma.

protect tumor cells from apoptosis rather than oncogenes that promote cell growth (Korsmeyer, 1992). Indeed, the anti-apoptotic genes *bcl-2*, *bcl-xL*, and *mcl-1* are expressed in CTCL cells, and their expression levels increase with progression of the disease (Zhang *et al.*, 2003). *Bcl-2* is a downstream target of STAT-3 signaling and is critical in controlling the activation of caspases by regulating the release of cytochrome *c* from the 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). In addition, survivin is a downstream target of NF- $\kappa$ B signaling and is a member of the inhibitor of apoptosis protein family (Ambrosini *et al.*, 1997). Survivin suppresses caspase activity and protects cells from apoptosis (LaCasse *et al.*, 1998). We have previously shown that CTCL cells express the survivin protein, and its expression decreases after bexarotene or avicin D treatment (Zhang *et al.*, 2002, 2008). In this study, curcumin treatment decreased protein and mRNA levels of both *bcl-2* and survivin in CTCL cell lines and in MF/SS patients' PBMCs. This suggests that the downregulation of *bcl-2* and survivin may be involved in curcumin-induced apoptosis in CTCL cells.

Curcumin has been shown to induce apoptosis through caspase-3-dependent or caspase-3-independent manner in a number of transformed tumor cells (Lopez-Lazaro, 2008). Caspase-3 is a key component of apoptosis, is activated in apoptotic cells, and cleaves cellular proteins, including PARP (Porter and Jänicke, 1999). Cleavage of PARP is a hallmark of apoptosis induced 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 after treatment with the RXR-selective retinoid bexarotene (Zhang *et al.*, 2002), and with the histone deacetylase inhibitor vorinostat (Zhang *et al.*, 2005). In this study, we show that curcumin treatment at concentrations inducing apoptosis also activated caspase-3 and cleaved PARP in CTCL cell lines and patients' PBMCs. Thus, curcumin-induced apoptosis is at least partially dependent on caspase-3 in CTCL cells. However, other types of cell death, in particular autophagy, may also be involved in curcumin-induced cell death in CTCL cells. Indeed, recent studies have shown that curcumin also induces autophagy, another type of programmed cell death (Lopez-Lazaro, 2008). It is interesting that our preliminary data showed that CTCL cells pretreated with the autophagy inhibitor CQ improved cell viability relative to cells treated with curcumin alone, suggesting that curcumin may also induce autophagy in CTCL. Further experiments for detecting autophagy such as autophagosomes are required to confirm our above observation in CTCL.

In conclusion, curcumin selectively induces apoptosis in CTCL cell lines and MF/SS patients' PBMCs compared with healthy donors' controls. These events are associated with downregulation of STAT-3, inhibition of DNA binding of NF- $\kappa$ B through suppression of phosphorylation of I $\kappa$ B- $\alpha$ , decreased mRNA and protein expression of *bcl-2* and survivin, and activation of caspase-3 in CTCL cells. Our

findings provide a mechanistic rationale for the potential use of curcumin as a therapeutic agent for patients with CTCL.

## MATERIALS AND METHODS

### Reagents

Curcumin (>95% pure) was purchased from LKT Laboratories (St Paul, MN). Curcumin was dissolved in dimethyl sulfoxide to a stock concentration of 20 mM and stored at  $-80^{\circ}\text{C}$ . Serial dilutions (5, 10, and 20  $\mu\text{M}$ ) were freshly prepared in RPMI 1640. CQ was purchased from Sigma-Aldrich (St Louis, MO) and was dissolved in phosphate-buffered saline to a stock concentration of 20 mM.

### Cells and cell culture

The human CTCL cell lines MJ (G11), Hut78, and HH, obtained from the American Type Culture Collection (Rockville, MD), were derived from the 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 eight MF/SS patients with 43.8–98.2% circulating CD4+CD26–tumor T cells (Bernengo *et al.*, 2001) (Table 1). Samples were obtained during routine diagnostic assessments. The Institutional Review Board of the University of Texas 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. PBMCs from these healthy donors and patients were isolated by Vacutainer CPT (Becton Dickinson, San Jose, CA). Cells were grown in RPMI 1640 medium (Sigma-Aldrich) 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%  $\text{CO}_2$  at  $37^{\circ}\text{C}$ .

### Cell viability

Cell viability was measured by CellTiter 96 AQueous One Solution Cell Proliferation Assay according to the manufacturer's instructions (Promega, Madison, WI). Aliquots of  $5 \times 10^4$  cells per well were distributed in 96-well microplates (Falcon, Franklin Lakes, NJ) in 100  $\mu\text{l}$  of medium and incubated with 5, 10, and 20  $\mu\text{M}$  curcumin or 10  $\mu\text{M}$  CQ for 24 and/or 48 hours, and then 20  $\mu\text{l}$  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 a 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 using an Annexin V-fluorescein isothiocyanate Detection Kit I (PharMingen, San Diego, CA) according to the manufacturer's instructions. Briefly, three cell lines (MJ, Hut78, and HH) and PBMCs from three healthy donors and eight MF/SS patients were incubated with or without curcumin (5, 10, and 20  $\mu\text{M}$ ) for 24 and/or 48 hours. Cells were collected, washed twice with cold phosphate-buffered saline, and centrifuged at 1,500 r.p.m. for 5 minutes. Cells were resuspended in  $1 \times$  binding buffer at a concentration of  $1 \times 10^6$  cells per ml, 100  $\mu\text{l}$  of the solution was transferred into a 5 ml culture tube, and 5  $\mu\text{l}$  of annexin V-fluorescein isothiocyanate and 5  $\mu\text{l}$  of PI (propidium iodide) were

added. Cells were gently vortexed, and incubated for 15 minutes at room temperature in the dark. Finally, 400  $\mu\text{l}$  of  $1 \times$  binding buffer was added to each tube, and samples were analyzed using FACScan flow cytometer (Becton Dickinson). For each sample, 10,000 ungated events were acquired. Annexin V<sup>+</sup>PI<sup>-</sup> cells represented the early apoptotic populations. Annexin V<sup>+</sup>PI<sup>+</sup> cells represented either late apoptotic or secondary necrotic populations.

### Isolation of cytoplasmic and nuclear extracts

Cells ( $5 \times 10^6$ ) 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  $\mu\text{l}$  of buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and  $1 \times$  protease inhibitor cocktail "complete mini" (Roche, Indianapolis, IN)). After a 20-minute 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  $\mu\text{l}$  of buffer C (10 mM HEPES (pH 7.9), 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 10% glycerol, and  $1 \times$  protease inhibitor cocktail "complete mini" (Roche)) and incubated at  $4^{\circ}\text{C}$  by shaking for 15 minutes. Protein concentrations were determined by the Bradford dye-binding protein assay (Bio-Rad, Richmond, CA) using bovine serum albumin as a standard.

### Western blot analysis

Western blotting was performed as described previously (Zhang *et al.*, 2002). Briefly, cytoplasmic (10  $\mu\text{g}$ ) or nuclear (5  $\mu\text{g}$ ) 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  $4^{\circ}\text{C}$  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-PARP (1:1,000), and anti-actin (1:2,000) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal mouse anti-phospho-STAT-3 (p-STAT-3) (1:1,000), anti- $\text{I}\kappa\text{B}-\alpha$  (1:1,000), anti-phospho- $\text{I}\kappa\text{B}-\alpha$  (p- $\text{I}\kappa\text{B}-\alpha$ ) (1:1,000), and rabbit anti-cleaved caspase-3 (1:1,000) antibodies were obtained from Cell Signaling (Beverly, MA). Immunoreactive bands were visualized using enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, UK), and equivalent loading of proteins in each well was confirmed by actin and Ponceau staining. The protein levels were quantified using an AlphaEase 4.0 Imager (Alpha Innotech, San Leandro, CA).

### Electrophoretic mobility-shift assay

For NF- $\kappa\text{B}$  binding activity, a nonradioactive electrophoresis mobility-shift assay kit was used according to the manufacturer's instructions (Panomics, Fremont, CA). Briefly, three CTCL cell lines and PBMCs from two MF/SS patients were incubated with or without curcumin (10  $\mu\text{M}$ ) for 24 hours. In all, 5  $\mu\text{g}$  of nuclear extracts was incubated  $15^{\circ}\text{C}$  for 30 minutes with a biotinylated oligonucleotide



containing the NF- $\kappa$ B binding site, and then the samples were separated on 6.0% nondenaturing polyacrylamide gel in  $0.5 \times$  TBE buffer for 55 minutes at 120V at 4°C. The gel contents were transferred into Biodyne B membrane (Pall, Ann Arbor, MI) for 45 minutes at 300 mA. The biotinylated nucleotides were detected using detected reagent (Panomics).

### Quantitative real-time PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The first-strand cDNA was synthesized from total RNA by reverse transcription using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Gene-specific quantitative real-time PCR assays for STAT-3, bcl-2, survivin, and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) (SABiosciences, Frederick, MD) were performed using a Biosystem 7000 sequencer (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. GAPDH was used as an internal standard of mRNA expression for normalization. Samples were run in triplicates with one control reaction containing no reverse-transcriptase enzyme to test for potential DNA contamination. Wells with no template were used as negative control. The results were expressed as mean  $\pm$  SD in three independent experiments.

### Statistical analysis

All experiments were performed in triplicate unless otherwise noted. Results were expressed as means  $\pm$  SD. Statistical significance was evaluated by Student's *t*-test, and  $P < 0.05$  was considered as significant.

### CONFLICT OF INTEREST

The authors state no conflict of interest.

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