Silibinin inhibits constitutive activation of Stat3, and causes caspase activation and apoptotic death of human prostate carcinoma DU145 cells

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Transcription factor signal transducer and activator of transcription (Stat)-3 is activated constitutively in prostate cancer (PCA) suggesting that its disruption could be an effective approach to control this malignancy. Here we assessed whether silibinin, a flavanone from *Silybum marianum* with proven anticancer efficacy in various cancer models, inhibits Stat3 activation in DU145 cells, and if it does, what is the biological fate of the cells? At 50 μM or higher concentrations for 24 or 48 h, silibinin concentration dependently reduced constitutive Stat3 phosphorylation at Tyr705 and Ser727 residues under both serum and serum-starved conditions. Constitutively active Stat3–DNA binding was also inhibited concentration dependently by silibinin; however, apoptotic death together with caspase and poly(ADP-ribose) polymerase (PARP) cleavage was observed by silibinin only under serum-starved conditions suggesting that additional survival pathways are active under serum conditions. In other studies, cells were treated with various specific pharmacological inhibitors where phosphorylation of Stat3 was not reduced by epidermal growth factor receptor and Mitogen activated protein/extracellular signal regulate kinase kinase (MEK1/2) inhibitors, suggesting lack of significant roles of these in Stat3 activation in DU145 cells. Janus kinase (JAK)-1 and JAK2 inhibitors strongly reduced Stat3 phosphorylation but did not result in apoptotic cell death. Interestingly, JAK1 inhibitor only in combination with silibinin resulted in a complete reduction in Stat3 phosphorylation at Tyr705, activated caspase-9 and caspase-3, and caused strong PARP cleavage and apoptotic death of DU145 cells. Given a critical role of Stat3 activation in PCA, our results showed that silibinin inhibits constitutively active Stat3 and induces apoptosis in DU145 cells, and thus might have potential significance in therapeutic intervention of this deadly malignancy.

Introduction

Prostate cancer (PCA) is the most common malignancy in elderly men in the USA, and is the second leading cause of cancer-related deaths in men following lung cancer (1). It is estimated that in 2006, there will be ~234,460 new cases of PCA in the United States, and ~27,350 men will die of this malignancy (1). The disease that has spread beyond the prostate is generally first treated with hormonal ablation (2,3), and cytotoxic chemotherapy is used in selected hormone-refractory patients (4,5). These treatment approaches, however, suffer from their own limitations together with the emergence of androgen independence during commonly used antiandrogen therapy (6). These limitations suggest that additional efforts must be devoted in developing novel agents that target the unique characteristics of the prostate carcinoma cells, specifically when this malignancy is progressed to advanced androgen-independent stage.

Abbreviations: EGFR, epidermal growth factor receptor; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; JAK, Janus kinase; PARP, poly(ADP-ribose) polymerase; PCA, prostate cancer; Stat, signal transducer and activator of transcription.

An aberrant activation of numerous signaling pathways, including both receptor and non-receptor tyrosine kinases, is a key element of cancer cell survival and growth (7). Consistent with this, several studies have shown that epidermal growth factor receptor (EGFR) and other members in its family together with the growth factors that activate them are over-expressed in both PCA tissues and derived cell lines, specifically at the advanced and androgen-independent stage of this malignancy (7). These observations suggest that targeting these molecular regulators and pathways of cancer cell growth and survival might help in achieving a resultant growth inhibition and death of cancerous cells. However, it is still to be determined if this targeting might also help in achieving a resultant growth inhibition and death of cancerous cells. The caveat, however, is that blocking all of them are not practically feasible, which leads to the step where most of them commonly converge, the transcription factors. Thus, targeting transcription factors holds more promise as it can even override the effect of aberrant signaling being transduced by upstream signaling molecules. Together, targeting transcription factors, which are inappropriately or constitutively active in cancer cells, seems to be a more effective approach for cancer intervention including PCA. Accordingly, research efforts are also being directed on targeting the activity and activation of transcription factors, specifically those that are inappropriately or constitutively active in cancer cells.

Materials and methods

Cell line and reagents

Human prostate carcinoma DU145 cell line was from American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 with 10% fetal
bovine serum (HyClone, Logan, UT) and 1% penicillin–streptomycin under standard culture conditions (37°C, 95% humidified air and 5% CO2). RPMI 1640 and other culture materials were from Invitrogen (Carlsbad, CA). Silibinin was purchased from Sigma Chemical Co. (St Louis, MO), and its purity was verified as 100% by high performance liquid chromatography as reported earlier (20). Phosphothreonin AG900 was from Alexis Biochemicals (San Diego, CA), and AG1478 and piceatannol were from Calbiochem (La Jolla, CA). Anti-cleaved caspase-3, anti-cleaved caspase-9, anti-cleaved poly(ADP-ribose) polymerase (PARP), anti-pStat3 (Tyr705), anti-pStat3 (Ser727), anti-Stat1 (Ser727), anti-Stat3 (Tyr694) and anti-Stat1 and anti-Stat3 antibodies were from Cell Signaling (Beverly, MA). Anti-Stat5 was obtained from Transduction Laboratories (Lexington, KY). Stat3 transactivation enzyme-linked immunosorbent assay kit (TransAM) was from Active Motif (Carlsbad, CA). Annexin V–Vibrant apoptosis kit was from Molecular Probes (Eugene, OR).

Cell culture and treatments
DU145 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum and 100 U/ml penicillin G and 100 μg/ml streptomycin sulfate under standard culture conditions. At 70% confluency, cells were treated with different concentrations of silibinin in DMSO or DMSO alone, either following 24 h of serum starvation or under 10% serum conditions. In the studies where cells were treated with specific inhibitor together with silibinin, inhibitor was added 2 h before the treatment with silibinin. After desired treatments, medium was aspirated and the cells were harvested by the addition of trypsin–EDTA, and as desired, total cell lysates and/or cytosolic extracts were prepared as described previously (22). Protein concentrations in lysates were determined using Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA) by the Lowry method.

Western immunoblot analysis
As desired, samples (30–80 μg protein) were denatured in 2× sample buffer, and were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 8, 12 or 16% Tris–glycine gels and separated proteins were transferred onto nitrocellulose membranes by western blotting. Membranes were blocked with blocking buffer for 1 h at room temperature, and probed with primary antibodies against desired molecules over night at 4°C followed by horseradish peroxidase-conjugated appropriate secondary antibody for 1 h at room temperature and proteins were visualized by enhanced chemiluminescence detection (Amerham Pharmacia Biotech, Piscataway, NJ). In each case, blots were subjected to multiple exposures on the X-ray film to ensure that the band density is in the linear range, and the bands were scanned with Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA).

Electrophoretic mobility shift assay and TransAM enzyme-linked immunosorbent assay
For electrophoretic mobility shift assay (EMSA), Stat3-specific oligonucleotide (3.5 pmol from Santa Cruz Biotechnology, Santa Cruz, CA) was end labeled with γ–32P-ATP using T4 polynucleotide kinase buffer as per vendor’s protocol (Promega, Madison, WI). The labeled double-stranded oligo probe was separated from free γ–32P-ATP using G-25 Sephadex column. To conduct the EMSA, 8 μg protein from nuclear extracts was incubated with 5× gel shift binding buffer (20% glycerol, 5 mM MgCl2, 2.5 mM EDTA, 2.5 mM diethiothreitol, 250 mM NaCl, 50 mM Tris–HCl and 0.25 mg/ml poly dI-dC) and then with 106 cpm end-labeled Stat3 consensus oligonucleotide for 20 min at 37°C. In competition assay, an unlabeled cold Stat3 oligo was co-incubated with labeled oligo, and for super-shift assay anti-Stat3 antibody was added in the assay mix before the addition of 106 cpm end-labeled Stat3 oligo. The incubation mixtures containing DNA–protein complex were resolved on 6% retardation gels (Invitrogen), and under the assay conditions, DNA–protein complexes were resolved on 6% retardation gels (Invitrogen), and then the gels were dried and bands were visualized by autoradiography.

TransAM method, introduced by Active Motif using the enzyme-linked immunosorbent assay (ELISA) to detect and quantify transcription factor activation was also used. Following desired treatments, cells were harvested and processed for Stat3 activation as described by manufacturer’s protocol. Briefly, DU145 cells at 60–70% confluency were treated with different concentrations of silibinin for 24 h, and nuclear extracts were prepared using the reagents provided in the kit and following vendor’s protocol. For each sample, 20 μg of nuclear extract protein was then diluted in complete lysis buffer and added into each well coated with oligonucleotide containing Stat consensus binding site. Under the assay conditions, Stats in nuclear extracts thus bind specifically to the oligonucleotide, and Stat3 is detected by using an antibody directed against Stat3 followed by a secondary antibody to horseradish peroxidase and colorimetric read out at 450 nm that is quantified by spectrophotometer.

Quantitative detection of apoptosis
To quantify silibinin-induced apoptotic death of DU145 cells, annexin V and propidium iodide staining was performed followed by flow cytometry, as described recently (19). Briefly, DU145 cells were plated in 60 mm dishes, and at ~50% confluency, cells were treated with different concentrations of silibinin without or with pre-treatment with specific inhibitor for 2 h. In case of serum-free conditions, cells plated for overnight (24 h) were switched to serum-free condition for another 24 h and then treated with silibinin under serum-free condition. After these treatments, cells were collected by brief trypsinization and washed with phosphate-buffered saline twice, and subjected to annexin V and propidium iodide staining using Vybrant Apoptosis Assay Kit2 following the step-by-step protocol provided by the manufacturer. After the staining, fluorescence-activated cell sorter analysis, utilizing the core service of the University of Colorado Cancer Center (Denver, CO), was performed for the quantification of the apoptotic cells.

Statistical analysis
Statistical significance of differences between control and treated samples were calculated by Student’s t-test (SigmaStat 2.03). P values of <0.05 were considered significant. Except mentioned otherwise, all the results are representative of at least two to four independent experiments with reproducible findings.

Results
Silibinin inhibits constitutively active Stat3 phosphorylation, and causes caspase activation and apoptosis
In our continued efforts to identify mechanisms and efficacy of sili-binin in human prostate carcinoma cells and the fact that PCA cells harbor constitutively active Stat3, first we assessed the effect of sili-binin on Stat3 phosphorylation. DU145 cells were grown to 70% confluency and then serum starved for 24 h followed by vehicle (control) or silibinin treatment at varying concentrations for 24 and 48 h. As shown in Figure 1A, strong levels of Tyr705- and Ser727-phosphorylated Stat3 were evidenced in 24 and 48 h vehicle controls that correspond to 48 and 72 h serum-starved DU145 cells, clearly suggesting constitutively active Stat3 levels in DU145 cells. Treatment of the cells under these conditions with silibinin resulted in a concentration- and a time-dependent decrease in both Tyr705- and Ser727-phosphorylated Stat3 without any noticeable changes in total Stat3 except at 200 μM silibinin concentration (Fig. 1A). Whereas lower silibinin concentration of 50 μM showed considerable effect, the higher concentration of 100 μM was very effective at 24 h with much stronger effect at 48 h in reducing the levels of both Tyr705- and Ser727-phosphorylated Stat3; the highest concentration of 200 μM silibinin showed almost complete disappearance of both Tyr705- and Ser727-phosphorylated Stat3 following 24 and 48 h treatment (Fig. 1A). Since we observed that silibinin treatment at highest concentration level of 200 μM led to a significant reduction in the total levels of Stat3, and Stat3 has been shown to be cleaved by activated caspases, we next assessed whether similar mechanism contributes to the observed decrease in total levels of Stat3 after silibinin treatment. We found that pre-treatment of DU145 cells with pan-caspase inhibitor, Z-VAD.fmk, failed to reverse the decrease in the phospho- and total levels of Stat3 (Fig. 1C), suggesting that the mechanisms other than caspase activation contribute in the observed alterations in Stat3.

The other important observation of this study was that serum starvation of DU145 cells up to 72 h in our study conditions did not result in any cell death including apoptosis (Fig. 1B), suggesting the possibility that constitutively active Stat3 provides a cell survival response in these cells. Silibinin treatment of these starved cells, however, resulted in a concentration- and a time-dependent apoptotic cell death where lower silibinin concentration of 50 μM showed no cell death, but the higher concentration of 100 μM caused a 3-fold increase (P < 0.01) versus vehicle control in apoptotic cells following 48 h treatment, and the highest concentration of 200 μM silibinin showed 5% (P < 0.001) and 28% (P < 0.001) apoptotic cells following 24 and 48 h treatment, which was 4- and 18-fold increase versus vehicle controls, respectively (Fig. 1B). In the studies examining whether the observed apoptotic response involves caspase activation, consistent with apoptotic cell death, silibinin also showed a concentration- and a time-dependent increase in caspase-3 and PARP cleavage (Fig. 1A). However, in case of caspase-9 cleavage, a concentration-dependent increase in its activation was observed only till 24 h of treatment time.
Since PCA development involves different stages such as hormone (androgen)-dependent followed by independent stages, we next studied the effect of siliibinin on the survival of different human PCA cell lines each representing a different stage of PCA, as well as on normal prostate cell line. As summarized in Table I, we observed that siliibinin treatment of serum-starved PC-3 cells, representing moderately aggressive stage of androgen-independent PCA, results in a significant apoptotic death from 4.5% in untreated controls to 18–32% (p < 0.02–0.001) at 50, 100 and 200 μM siliibinin for 24 h. Under serum conditions, a significant apoptotic death (15.6%) was observed only at highest concentration of 200 μM siliibinin. However, in case of androgen-dependent LNCaP cells, treatment with siliibinin for 24 h, both under serum and serum-starved conditions resulted in only 5–6% apoptotic cell population that too at the highest concentration of 200 μM. A similar response was also observed in human normal prostate cell line, PWR-1E, under serum conditions; however, when these cells were serum starved, strong apoptotic effect of siliibinin was evidenced at all the concentrations (Table I).

**Siliibinin inhibits Stat3 phosphorylation in DU145 cells.**

Together, the results shown in Figure 1 suggested that an inhibition of constitutively active Stat3 by siliibinin might be responsible for the observed caspase activation and associated apoptotic death of DU145 cells. These apoptotic effects of siliibinin in DU145 cells, however, were in contrast to our earlier findings where we have shown that siliibinin treatment of DU145 cells causes cell growth inhibition and DNA synthesis inhibition, but not apoptotic cell death (23). The only difference between previous studies and the present work is serum conditions, and therefore, we next asked the question whether siliibinin affects Stat3 phosphorylation in DU145 cells under serum conditions, and if so, what would be the biological fate of the cells.

DU145 cells at 70% confluency without any serum starvation were treated with vehicle or varying concentrations of siliibinin, and after 24 and 48 h, total cell lysates were analyzed for Stat3 phosphorylation at both Tyr705 and Ser727 residues. As shown in Figure 2A, consistent with the observations made under serum-starved condition, siliibinin caused a concentration-dependent decrease in the levels of both Tyr705- and Ser727-phosphorylated Stat3; however, unlike under serum-starved conditions, there was only a minimal decrease in total Stat3 following highest concentration of siliibinin treatment. To further support our observations that siliibinin indeed inhibits Stat3 activation, additional studies were done where first we analyzed the levels of both Tyr705- and Ser727-phosphorylated Stat3 in the cytosolic and nuclear fractions prepared from siliibinin-treated cells under identical conditions. As shown in Figure 2B, siliibinin showed a concentration-dependent decrease in the levels of Tyr705-phosphorylated Stat3, without any measurable effect on Ser727-phosphorylated and total Stat3.

**Siliibinin inhibits Stat3 activation under serum condition without apoptosis induction**

**Table 1. Extent of apoptotic death induced by siliibinin in different prostate cell lines**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>% Apoptotic cell death</th>
</tr>
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<tbody>
<tr>
<td>10% Serum (24 h)</td>
<td>Serum starved (24 h)</td>
<td></td>
</tr>
<tr>
<td>PC-3</td>
<td>Control</td>
<td>2.83 ± 0.13</td>
</tr>
<tr>
<td>50 μM Sb</td>
<td>2.86 ± 0.69</td>
<td>20.66 ± 5.84***</td>
</tr>
<tr>
<td>100 μM Sb</td>
<td>3.46 ± 0.49</td>
<td>17.90 ± 0.47**</td>
</tr>
<tr>
<td>200 μM Sb</td>
<td>15.57 ± 2.44***</td>
<td>32.30 ± 2.5***</td>
</tr>
<tr>
<td>LNCaP</td>
<td>Control</td>
<td>2.81 ± 0.45</td>
</tr>
<tr>
<td>50 μM Sb</td>
<td>3.98 ± 0.67</td>
<td>4.6 ± 0.56</td>
</tr>
<tr>
<td>100 μM Sb</td>
<td>2.99 ± 0.45</td>
<td>4.3 ± 0.34</td>
</tr>
<tr>
<td>200 μM Sb</td>
<td>6.13 ± 0.06***</td>
<td>5.49 ± 0.08**</td>
</tr>
<tr>
<td>PWR-1E</td>
<td>Control</td>
<td>1.23 ± 0.15</td>
</tr>
<tr>
<td>50 μM Sb</td>
<td>2.26 ± 0.20</td>
<td>18.22 ± 0.05*</td>
</tr>
<tr>
<td>100 μM Sb</td>
<td>4.43 ± 0.27***</td>
<td>44.74 ± 4.82***</td>
</tr>
<tr>
<td>200 μM Sb</td>
<td>5.62 ± 0.65***</td>
<td>40.97 ± 3.2***</td>
</tr>
</tbody>
</table>

PC-3, LNCaP or PWR-1E cells with or without serum starvation for 24 h were treated either with DMSO (control) or siliibinin (Sb) at 50–200 μM concentrations for 24 h. At the end of treatment time, cells were collected and processed for annexin V–propidium iodide staining followed by fluorescence-activated cell sorter analysis. The data shown in each case are mean of three independent samples with SE.

*P < 0.05, **P < 0.01, ***P < 0.001, versus DMSO control.

**Fig. 1. Effect of siliibinin on constitutively active Stat3 phosphorylation and apoptosis induction.** DU145 cells were serum starved for 24 h and treated with either DMSO (control, C) or different concentrations of siliibinin for 24 and 48 h. At the end of these treatments, (A) cell lysates were prepared and analyzed by western blotting for phospho (Tyr705 and Ser727) and total Stat3, cleaved caspase-9, caspase-3 and PARP, and protein loading was checked by stripping and re-probing the membranes for β-actin, and (B) cells were harvested and processed for flow cytometric analysis of annexin V–propidium iodide-stained apoptotic cells as described in Materials and Methods. Quantitative data are presented as mean ± SE of triplicate samples, which were reproducible in an additional independent experiment. P < 0.01 and P < 0.001 as compared with DMSO-treated control for each treatment time. (C) DU145 cells were serum starved for 24 h and then treated with 200 μM siliibinin for 24 and 48 h with or without pre-treatment with 50 μM Z-VAD.fmk for 2 h. Total cell lysates were prepared at the end of treatment times, and were analyzed by western blotting for phospho (Tyr705 and Ser727) and total Stat3. Sb, siliibinin.

**Fig. 2. Effect of siliibinin on constitutively active Stat3 phosphorylation and apoptosis induction.** DU145 cells were serum starved for 24 h and then treated with 200 μM siliibinin for 24 and 48 h. Under serum conditions, a significant apoptotic death (15.6%) was observed only at highest concentration of 200 μM siliibinin. However, in case of androgen-dependent LNCaP cells, treatment with siliibinin for 24 h, both under serum and serum-starved conditions resulted in only 5–6% apoptotic cell population that too at the highest concentration of 200 μM. A similar response was also observed in human normal prostate cell line, PWR-1E, under serum conditions; however, when these cells were serum starved, strong apoptotic effect of siliibinin was evidenced at all the concentrations (Table I).
Stat3 levels in the cytosolic fractions. However, the levels of both Tyr705- and Ser727-phosphorylated Stat3 decreased concentration dependently by silibinin in the nuclear fraction without much effect on total Stat3 (Fig. 2C).

Qualitative EMSA and quantitative ELISAs were next performed to further examine the effect of silibinin on Stat3 activation. Following treatment of DU145 cells with silibinin under identical conditions as for other studies in Figure 2 including 10% serum condition, equal amount of protein from nuclear extract was used for assessing Stat3 activation by EMSA. Compared with vehicle control, silibinin inhibited constitutive activation of Stat3 following 24 h of its treatment in a concentration-dependent manner (Fig. 3A). In the studies analyzing the specificity of Stat3 bands marked with arrows (Fig. 3A) possibly representing Stat3 homodimer and heterodimer (with other Stats) (24), addition of unlabeled Stat3 probe in EMSA incubation resulted in a disappearance of these bands (Fig. 3A). Additionally, super-shift assay was performed to support the validity of marked bands for activated Stat3 forms where nuclear extracts were first incubated with anti-Stat3 antibody followed by EMSA. This also showed a disappearance of marked bands suggesting them to be activated Stat3 forms (Fig. 3A). The qualitative EMSA observations were further supported by quantitative TransAM Stat3 ELISA, where compared with vehicle control, silibinin treatment at 100 and 200 μM concentrations for 24 h resulted in 30% (P < 0.01) and 65% (P < 0.001) decrease in constitutively active Stat3 in DU145 cells, respectively (Fig. 3B). Based on the effects of silibinin on Stat3 phosphorylation and total levels in DU145 cells, we next examined whether it also affects other members of the Stat family. As shown in Figure 3C, silibinin treatment caused concentration-dependent decrease in the phosphorylation of Stat1 at Ser727 residue under both serum- and serum-starved conditions without affecting the total levels of Stat1. However, the phosphorylated levels of Stat1 at Tyr701 residue were not detectable under these experimental conditions. Similarly, we could not detect the phosphorylated
levels of Stat2 in this cell line (data not shown). Contrary to all other results, a significant increase in the phosphorylated levels of Stat3 at Tyr694 without any effect on the total levels of Stat3 was observed upon silibinin treatment of DU145 cells cultured under both serum starved and 10% serum conditions (Fig. 3C).

The results shown in Figures 2 and 3 clearly demonstrate that silibinin treatment of DU145 cells under 10% serum condition concentration dependently decreases the phosphorylation of Stat3 at both Tyr705 and Ser727 sites (Fig. 2), and that silibinin inhibits constitutively active Stat3 activation (Fig. 3); however, under these treatment conditions, we did not observe any cell death and apoptotic effect of silibinin in DU145 cells (data not shown) as opposed to strong apoptotic effect shown in Figure 1B when experiments were done under serum-starved conditions. Considering these observations together, one possibility was that under serum condition, there is a survival signal, which is not affected by silibinin, and the resultant biological response is a lack of apoptosis even though Stat3 activation is inhibited by silibinin. Accordingly, next we examined the effect of silibinin on Stat3 phosphorylation and apoptosis in presence of the inhibitors for EGFR, extracellular signal regulated kinase (ERK1/2), JAK1 and JAK2.

Effect of EGFR and MEK1/2 inhibitors on Stat3 phosphorylation

Both EGFR and ERK1/2 have been shown to be constitutively active in advanced and androgen-independent human PCA and derived cell lines including DU145 cells, and it has been well established that activation of these molecules correlate with PCA progression together with higher Gleason grade (25). Accordingly, first we asked the question whether activation of EGFR or ERK1/2 provides a survival mechanism in DU145 cell treatment with silibinin under serum conditions. To address this question, cells were treated first with varying concentrations of EGFR inhibitor AG1478, and Stat3 phosphorylation was examined. AG1478 treatment did not result in any change in Stat3 phosphorylation at Tyr705, but caused a modest decrease in Stat3 phosphorylation at Ser727 without any change in total Stat3 in lower concentrations but a modest decrease at the highest concentration of 1 \( \mu M \) (Fig. 4A). Studies were next performed to assess the effect of AG1478 in combination with silibinin on Stat3 phosphorylation and apoptotic cell death. As shown in Figure 4B, even a higher concentration of AG1478 (2 \( \mu M \)) did not produce any effect of Stat3 phosphorylation; however, consistent with the findings shown in Figure 2, silibinin alone and in combination with varying concentrations of AG1478 caused a very strong decrease in Stat3 phosphorylation at Tyr705 together with modest decrease in Ser727 phosphorylation (Fig. 4B). In terms of apoptotic effects, neither AG1478 alone at varying concentrations nor their combinations with silibinin produce any apoptotic effect of DU145 cells (data not shown). In the studies where a MEK1/2 inhibitor PD98059 was used to inhibit ERK1/2 activation, there was no decrease in Stat3 phosphorylation at Tyr705, but again a modest decrease in Ser727 phosphorylation occurred without any changes in total Stat3 (Fig. 4C). Silibinin alone caused a strong decrease in Stat3 phosphorylation at Tyr705 and Ser727, which did not change in the presence of PD98059 (Fig. 4C). In terms of apoptotic effects, again there was no apoptotic cell death following PD98059 treatment either alone or in silibinin combination (data not shown), even though the concentrations of these inhibitors (AG1478 and PD98059) used in our study effectively inhibited the phosphorylation of their targets (Figs. 4D and E). Together, these results suggest that EGFR and ERK1/2 activation does not play a major role in Stat3 phosphorylation as well as towards the apoptotic effect of silibinin in DU145 cells under serum conditions.

Effect of JAK1 and JAK2 inhibitors on Stat3 phosphorylation and apoptosis in presence of silibinin

Cytokines and growth factors in serum are also known to activate Stats via Janus kinase (JAK) activation (24), and therefore, next we assessed the effect of JAK1 and JAK2 inhibitors on Stat3 phosphorylation and apoptotic death of DU145 cells under serum conditions without or with silibinin treatment. Treatment of DU145 cells with JAK1 inhibitor piceatannol alone at 50 \( \mu M \) concentration for 24 h did not show any effect on Stat3 phosphorylation at both Tyr705 and Ser727 sites; however, a higher concentration of 100 \( \mu M \) caused a strong reduction in Tyr705 phosphorylation with modest effect on Ser727 (Fig. 5A). A higher treatment time of 48 h also showed similar responses (data not shown). Similar to the effect of JAK1 inhibitor, when cells were treated with JAK2 inhibitor tyrphostin (AG490) under identical treatment conditions, only higher concentration AG490 showed a reduction in Stat3 phosphorylation at Tyr705 even at 100 \( \mu M \) concentrations of piceatannol and AG490, and that Stat3 phosphorylation at Ser727 is only modestly, if at all, decreased by these inhibitors. In terms of their effect on apoptosis induction, we
phosphorylation at Tyr705 was evidenced and there was no apoptotic cell death. When cells were treated with lower concentration (50 μM) of piceatannol and AG490 together with silibinin for 24 and 48 h, no additional decrease in Stat3 phosphorylation to drive apoptotic cell death. When cells were treated with JAK1 inhibitor (piceatannol) or JAK2 inhibitor (AG490) at a concentration (100 μM) for an additional 22 h. At the end of these treatments, total cell lysates were prepared and western blotting was carried out for pStat3 (Tyr705 and Ser727) and total Stat3, and cleaved caspase-9, caspase-3 and PARP; protein loading was checked by stripping and re-probing the same membranes for β-actin in each case. In other similar treatments, cells were harvested and processed for flow cytometric analysis of annexin V–propidium iodide-stained apoptotic cells as described in Materials and Methods. Quantitative data shown in panel (B) are presented as mean ± SE of triplicate samples, which were reproducible in two additional independent experiments. P < 0.001 as compared with DMSO-treated control. Sb, silibinin; Pic, piceatannol.

Fig. 5. Effect of JAK1 and JAK2 inhibitors on Stat3 phosphorylation and apoptosis in presence of silibinin. DU145 cells without any serum starvation were treated with JAK1 inhibitor (piceatannol) or JAK2 inhibitor (AG490) for 2 h then treated with or without silibinin (100 μM) for an additional 22 h. At the end of these treatments, total cell lysates were prepared and western blotting was carried out for (A) pStat3 (Tyr705 and Ser727) and total Stat3, and (C) cleaved caspase-9, caspase-3 and PARP; protein loading was checked by stripping and re-probing the same membranes for β-actin in each case. In other similar treatments, cells were harvested and processed for flow cytometric analysis of annexin V–propidium iodide-stained apoptotic cells as described in Materials and Methods. Quantitative data shown in panel (B) are presented as mean ± SE of triplicate samples, which were reproducible in two additional independent experiments. P < 0.001 as compared with DMSO-treated control. Sb, silibinin; Pic, piceatannol.

did not see much change in annexin V–propidium iodide staining as well as cleavage of caspase-9, caspase-3 and PARP following treatment of cells at 100 μM concentration of piceatannol and AG490 alone for 24 h (Fig. 5B and C).

Together, these results suggested that JAK inhibitors reduce Stat3 phosphorylation at Tyr705; however, this effect is not complete to possibly induce an apoptotic death of DU145 cells under serum conditions. Accordingly, we next asked the question whether silibinin combination with these JAK inhibitors produces better effects on Stat3 phosphorylation to drive apoptotic cell death. When cells were treated with lower concentration (50 μM) of piceatannol or AG490 together with silibinin for 24 and 48 h, no additional decrease in Stat3 phosphorylation at Tyr705 was evidenced and there was no apoptotic cell death (data not shown). However, treatment of cells with higher concentration (100 μM) of JAK1 inhibitor piceatannol together with silibinin for 24 h resulted in a complete reduction in Stat3 phosphorylation at Tyr705 compared with each agent alone (Fig. 5A). The most important observation, when we employed this combination, was a strong apoptotic death of DU145 cells together with strong levels of cleaved caspase-9, caspase-3 and PARP (Fig. 5B and C). In case of identical combination studies with JAK2 inhibitor AG490, whereas stronger decrease in Stat3 phosphorylation at both Tyr705 and Ser727 sites was evident, there was not a complete reduction in Tyr705 phosphorylation of Stat3 as in case of JAK1 inhibitor piceatannol plus silibinin (Fig. 5A). Consistent with these findings, AG490 combination with silibinin did not show apoptotic effect though a modest increase in cleaved caspase-9, caspase-3 and PARP was observed when compared with control or each agent alone. Together, these observations suggest that a complete reduction in Stat3 phosphorylation at Tyr705 possibly is an important event for the apoptotic death of DU145 cells under serum conditions. We would also like to mention here that identical treatments of DU145 cells shown in Figure 5 for 48 h did not produce major changes to those shown for 24 h in this figure, except piceatannol combination with silibinin (each at 100 μM concentration) caused almost 90% apoptotic cell death and that piceatannol (50 μM) plus silibinin (100 μM) also showed 20% apoptotic cell death at this treatment time of 48 h as opposed to no such effect following 24 h treatment (data not shown).

**Effect of silibinin on downstream targets of Stat3 activation**

Constitutive activation of Stat3 regulates cell cycle progression by affecting cell cycle regulatory molecules such as up-regulation of cyclin D (1, 2 and 3) and down-regulation of the expression of p21 and p27. It also activates the anti-apoptotic signals by regulating the expression of Bcl-XL, Mcl-1 and survivin. We, therefore, next tried to study the effect of silibinin on the expression of these cell cycle progression and anti-apoptotic molecules. We observed that silibinin treatment of DU145 cells cultured under serum-starved condition down-regulated the protein levels of cyclin D1, Mcl-1, Bcl-XL and survivin in a concentration-dependent manner (Fig. 6). This might explain our initial observation that silibinin induces apoptotic death in serum-starved DU145 cells.

**Discussion**

PCA control and treatment employing conventional therapeutic approaches have had limited success (26), and therefore, several efforts are being made to identify and develop new agents for both intervention and prevention of PCA (27). One such agent that has received significant attention in recent years is silibinin which exhibits no toxicity and/or any substantial adverse effects in both animal studies and clinical trials, and has shown strong anticancer and chemopreventive effects in various in vitro and in vivo cancer models (16–20).

Based on these facts, employing silibinin treatments in advanced and androgen-independent human prostate carcinoma DU145 cells, the central finding of the present study is that this agent strongly inhibits constitutive activation of Stat3, and causes caspase activation and apoptotic death of DU145 cells. However, in another androgen-independent human prostate carcinoma PC-3 cells, silibinin treatment showed only a moderate apoptotic effect probably because of impaired Stat3 signaling in these cells. Similarly, androgen-dependent LNCaP cells, which do not have constitutively activated Stat3 signaling, were resistant to silibinin-induced apoptotic death as compared with DU145 cells. These results suggest that the observed biological effects of silibinin in these PCA cells might have an association with their Stat3 signaling status.

Stats comprises signaling molecules that regulate the expression of genes by modulating their transcription in response to various stimuli including growth factors and cytokines (28). They were first identified as transcription factors, which are activated by interferon, and are currently part of signaling cascades for several growth factors and...
cancer and tumorigenicity. And accordingly Stat3 blockade by various approaches has been the induction of apoptosis in tumor cells including prostate carcinoma, and accordingly Stat3 blockade by various approaches has been shown to result in progression of this malignancy (8,11,45). In this regard, disruption of over-expression in human PCA, suggesting its involvement in the literature reports showing constitutively active Stat3 as well as its future to address this issue.

For silibinin to exert its apoptotic effect. More studies are needed in combination, we suggest that under serum condition, there is a substantial role in Stat3 phosphorylation in DU145 cells as EGFR and platelet-derived growth factor receptor (32,33). For their biological activity, Stats are phosphorylated at tyrosine and serine residues by upstream non-receptor tyrosine kinases Src and JAKs, as well as by growth factor receptors such as EGFR and platelet-derived growth factor receptor (32,33). Whereas activation of Stats is a tightly regulated event in normal/benign cells, an aberrant activation is observed in malignancies of both hematopoietic and non-hematopoietic origin such as myeloma, and head and neck, breast and PCA (34,35). Further, malignant cells with constitutively activated Stat3 are reported as self-dependent for their survival (36). Downstream target genes of active Stat3 include cyclin D1 and D2, c-myc, p53, Bcl-XL, Bcl-2, Mcl-1, survivin and vascular endothelial growth factor (37–42). Deregulated expression of these target genes influences cell cycle progression, apoptosis and angiogenesis. In present study, we have shown that silibinin inhibits constitutively active Stat3 phosphorylation and Stat3–DNA binding along with down-regulation of cyclin D1, Bcl-XL, Mcl-1 and survivin. Down-regulation of Bcl-XL might be responsible for apoptosis induction in DU145 cells consistent with the earlier report in literature wherein down-regulation of Bcl-XL as a consequence of disruption of Stat3 signaling resulted in the induction of apoptosis (8). In addition, Stat3 down-regulation might be a contributory factor to the G1 arrest induced by silibinin in another androgen-independent cell line, PC-3, wherein down-regulation of cyclins D1 and D3 was also observed (18). Numerous studies have also implicated mitogen activated protein kinase (MAPK) family members in the phosphorylation and subsequently the activation of Stat3 (43). Additionally, silibinin has been shown to reduce the invasion of A549 cells via the suppression of Akt and/or ERK1/2 phosphorylation (44). However, our results showed that EGFR pathway and/or ERK1/2 pathway does not play any substantial role in Stat3 phosphorylation in DU145 cells as EGFR and MEK1/2-specific inhibitors were not able to reduce Stat3 phosphorylation at Tyr705 and/or Ser277 sites. Furthermore, based on our results with JAK1 and JAK2 inhibitors without and with silibinin combination, we suggest that under serum condition, there is a survival pathway downstream of JAK1 that needs to be inhibited in order for silibinin to exert its apoptotic effect. More studies are needed in future to address this issue.

The significance of our present findings also associates with the literature reports showing constitutively active Stat3 as well as its over-expression in human PCA, suggesting its involvement in the progression of this malignancy (8,11,45). In this regard, disruption in the activation of Stat3 or its expression has been shown to result in the induction of apoptosis in tumor cells including prostate carcinoma, and accordingly Stat3 blockade by various approaches has been employed to suppress the proliferation of various human cancer cells in culture and tumorigenicity in vivo (10,38,46–48). Once again, these studies also suggest that targeting constitutively active Stat3 by chemopreventive agents could be a promising approach for PCA intervention; however, only limited efforts have been made in this direction. For example, the derivatives of indirubin, an active compo-

nent of Chinese herbal medicine, were found to potently block constitutively active Stat3 in human breast and PCA cells by inhibiting Src kinase activity and Stat3–DNA binding activity (49). Zylamlin, which is also a non-selective COX inhibitor, has been reported to inhibit Stat3 phosphorylation in LNCaP cells (50). Another study by Kotha et al. (12) reported that resveratrol causes cell cycle arrest and apoptosis in DU145 cells and represses Stat3-regulated cyclin D1, Bcl-XL, and Mcl-1 genes. Consistent as well as in continuation with these findings, in the present study, we demonstrate that silibinin inhibits constitutive activation of Stat3 and causes caspase activation and apoptotic death of DU145 cells. The results of the present study might have important implications in altering the outcome of PCA in humans because of high bioavailability of silibinin in human plasma, as in our ongoing clinical trial (20), we were able to achieve nearly 70 µM concentration of circulating silibinin in humans when it was given to patients in the form of silibinin phytosome.

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References


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