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.

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 (21). Pdidothepin AG990 were 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 translocation assay kit (TransAM) was from Active Motif (Carlsbad, CA). Annexin V–Vybrant 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% confluence, 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 then washed by trypsin EDTA (5.0 mM EDTA, 0.5 mM Tris–HCl) and resuspended in 0.25% trypsin–EDTA and then 50% confluency, cells were treated with different concentrations of silibinin described recently (19). Briefly, DU145 cells were plated in 60 mm dishes, and at ~50% confluence, 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 shown 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% confluence 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 (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 silibinin 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 silibinin 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 $\mu$M silibinin for 24 h. Under serum conditions, a significant apoptotic death (15.6%) was observed only at highest concentration of 200 $\mu$M silibinin. However, in case of androgen-dependent LNCaP cells, treatment with silibinin 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 $\mu$M. 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 silibinin was evidenced at all the concentrations (Table I).

### Silibinin inhibits Stat3 activation under serum condition without apoptosis induction

Together, the results shown in Figure 1 suggested that an inhibition of constitutively active Stat3 by silibinin might be responsible for the observed caspase activation and associated apoptotic death of DU145 cells. These apoptotic effects of silibinin in DU145 cells, however, were in contrast to our earlier findings where we have shown that silibinin 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 silibinin 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 silibinin, 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, silibinin 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 silibinin treatment. To further support our observations that silibinin 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 silibinin-treated cells under identical conditions. As shown in Figure 2B, silibinin showed a concentration-dependent decrease in the levels of Tyr705-phosphorylated Stat3, without any measurable effect on Ser727-phosphorylated total Stat3.

### Table I. Extent of apoptotic death induced by silibinin in different prostate cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>% Apoptotic cell death</th>
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<tbody>
<tr>
<td></td>
<td>10% Serum (24 h)</td>
<td>Serum starved (24 h)</td>
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<tr>
<td>PC-3</td>
<td>Control</td>
<td>2.83 ± 0.13</td>
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<tr>
<td></td>
<td>50 $\mu$M Sb</td>
<td>2.86 ± 0.69</td>
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<tr>
<td></td>
<td>100 $\mu$M Sb</td>
<td>3.46 ± 0.49</td>
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<tr>
<td></td>
<td>200 $\mu$M Sb</td>
<td>15.57 ± 2.44***</td>
</tr>
<tr>
<td>LNCaP</td>
<td>Control</td>
<td>2.81 ± 0.45</td>
</tr>
<tr>
<td></td>
<td>50 $\mu$M Sb</td>
<td>3.98 ± 0.67</td>
</tr>
<tr>
<td></td>
<td>100 $\mu$M Sb</td>
<td>2.99 ± 0.45</td>
</tr>
<tr>
<td></td>
<td>200 $\mu$M Sb</td>
<td>6.13 ± 0.06**</td>
</tr>
<tr>
<td>PWR-1E</td>
<td>Control</td>
<td>1.23 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>50 $\mu$M Sb</td>
<td>2.26 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>100 $\mu$M Sb</td>
<td>4.43 ± 0.27**</td>
</tr>
<tr>
<td></td>
<td>200 $\mu$M Sb</td>
<td>5.62 ± 0.65**</td>
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</table>

PC-3, LNCaP or PWR-1E cells with or without serum starvation for 24 h were treated either with DMSO (control) or silibinin (Sb) at 50–200 $\mu$M 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.
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 Stat3 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

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**Fig. 2.** Effect of silibinin on Stat3 phosphorylation under serum condition. DU145 cells without any serum starvation were treated with either DMSO (control, C) or different concentrations of silibinin for 24 and 48 h. At the end of these treatments, whole-cell lysates (A), and cytosolic (B) and nuclear (C) extracts were analyzed by western blotting for phospho (Tyr705 and Ser727) and total Stat3, and protein loading was checked by stripping and re-probing the same membranes for β-actin and histone H1. Sb, silibinin.

**Fig. 3.** Effect of silibinin on constitutively active Stat3–DNA binding. DU145 cells without any serum starvation were treated for 24 h with either DMSO (control) or 50, 100 and 200 μM concentrations of silibinin. After these treatments, nuclear extract were prepared, and (A) EMSA was performed for Stat3–DNA binding activity wherein we observed two bands being reduced following silibinin treatments, possibly representing Stat3/3 (upper) and Stat3/1 (lower) dimers, or (B) ELISA was performed to detect and quantify Stat3 activation, as described in Material and Methods. Quantitative data in panel (B) are presented as mean ± SE of triplicate samples. $P < 0.01$ and $P < 0.001$ as compared with DMSO-treated control. (C) DU145 cells with and without serum starvation for 24 h were treated for another 24 h with either DMSO (control) or 50, 100 and 200 μM concentrations of silibinin. After these treatments, total extracts were prepared and analyzed by western blotting for phosphoStat5 (Tyr694) and total Stat5, phospho Stat1 (Ser727) and Stat1 protein levels. Sb, silibinin
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 μM (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 μM) 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 death 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 μM concentration for 24 h did not show any effect on Stat3 phosphorylation at both Tyr705 and Ser727 sites; however, a higher concentration of 100 μM 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 tyrophostin (AG490) under identical treatment conditions, only higher concentration AG490 showed a reduction in Stat3 phosphorylation at Tyr705 (Fig. 5A). The important observation from these JAK inhibitor studies was that we did not see a complete reduction in Stat3 phosphorylation at Tyr705 even at 100 μM 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
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 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. (B) 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.

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 cells 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.
Silibinin inhibits Stat3 in prostate cancer

cytokines (29–31). 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 Ser727 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-