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Isosilybin A Induces Apoptosis in Human Prostate Cancer Cells via Targeting Akt, NF- κ B and Androgen Receptor Signaling

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Abstract

Prostate cancer (PCA) is second most malignancy in American men. Advanced stage PCA cells possess unlimited replication potential as well as resistance to apoptosis. Therefore, targeting survival mechanisms and activating apoptotic machinery in PCA cells using non-toxic phytochemicals is suggested as an attractive strategy against this deadly malignancy. In the present study, we assessed the effect of one such botanical agent, namely isosilybin A, on apoptotic machinery and key members of cell survival signaling [Akt, NF- κ B and androgen receptor (AR)] in different PCA cells. Results showed that isosilybin A (90–180 μ M) treatment significantly induces apoptotic death by activating both extrinsic (increased level of DR5 and cleaved caspase 8) and intrinsic pathways (caspase 9 and caspase 3 activation) of apoptosis in three different human PCA cell lines namely 22Rv1, LAPC4 and LNCaP. Further, isosilybin A treatment decreased the levels of phospho-Akt (Serine-473), total Akt and the nuclear levels of NF- κ B constituents (p50 and p65). Isosilybin A treatment also decreased the AR and PSA level in 22Rv1, LAPC4 and LNCaP cells. Employing pan-caspase inhibitor (Z-VAD.fmk), we confirmed that isosilybin A-mediated decreased AR is independent of caspases activation. Temporal kinetics analysis showed that the primary effect of isosilybin A is on AR, as decrease in AR was evident much earlier (4h) relative to caspase activation and apoptosis induction (12h). Overall, our results demonstrated that isosilybin A activates apoptotic machinery in PCA cells via targeting Akt-NF- κ B-AR axis; thereby, indicating a promising role for this phytochemical in the management of clinical PCA.

Keywords

Prostate cancer; isosilybin A; chemoprevention; apoptosis; androgen receptor

INTRODUCTION

Prostate cancer (PCA) is the most commonly diagnosed non-cutaneous neoplasm, and is the second leading cause of cancer-related deaths in American men after lung cancer [1].

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According to the American Cancer Society, about 192,280 cases and 27,360 deaths due to PCA were estimated in American men in 2009 [1]. The currently available therapies such as radiotherapy, chemotherapy, and hormonal therapy seem inadequate and non-effective towards advanced stage PCA cells, and toxic towards normal tissues of the body; therefore, alternative measures are warranted for the better management of PCA. Prevention and therapeutic intervention by phytochemicals is an attractive alternative, as their efficacy, human acceptance and non-toxicity is supported by epidemiological and experimental evidences [2–4]. In this regard, the cancer chemopreventive and therapeutic role of silymarin (isolated from the seeds of milk thistle, *Silybum marianum*) has been extensively reported against various cancers including PCA [5,6].

Silymarin is a mixture of various polyphenols, consisting of seven distinct flavonolignans, namely silybin A, silybin B, isosilybin A, isosilybin B, silydianin, silychristin, isosilychristin; and one flavonoid taxifolin [7–9]. In recent studies, we compared the biological effect of these pure compounds on various anti-proliferative end points, including prostate cancer cell growth suppression, cell cycle distribution, clonogenic potential, inhibition of AR signaling, and DNA topoisomerase II α promoter activity [8,10–12]. In these studies, isosilybin A was identified as one of the biologically active constituents of silymarin with strong propensity to induce cell death in PCA cells [10,13].

Cell survival is maintained by a delicate balance between anti-apoptotic and pro-apoptotic stimuli. Dysregulation of this balance could disrupt the equilibrium between cell proliferation and cell death, and it is considered an essential event in the development and progression of cancer [14]. In general, apoptosis is activated through two major signaling pathways, namely the intrinsic and extrinsic pathways [15–18]. The intrinsic apoptotic pathway is triggered in response to DNA damage or other types of severe cell stress and involves the release of cytochrome c from the inter-membrane space of the mitochondria [15–18]. The released cytochrome c interacts with APAF1 (apoptotic protease-activating factor 1) and forms an “apoptosome” that activates caspase 9, leading to the activation of downstream caspases (3, 6, and 7) and the apoptotic death response [15–18]. The extrinsic pathway of apoptosis is triggered in response to pro-apoptotic ligands [TRAIL (Tumor necrosis factor-related apoptosis-inducing ligand), TNF (Tumor necrosis factor), Fas ligands etc], which bind and activate specific pro-apoptotic death receptors [death receptor (DR) 4, DR5, TNFR (TNF receptor), Fas etc] [17,18]. Ligand-induced activation of death receptors leads to the recruitment of the adaptor Fas-associated death domain (FADD) and activation of protease caspases 8 [15,17,18]. Caspase 8 cleaves and activates caspase 3 as well as other downstream caspases, thereby triggering apoptosis [15,17,18]. Cancer cells with alterations in the molecules involved in the above mentioned cell death signaling are often resistant to radio- and chemo-therapy that acts primarily through induction of apoptosis [16–18]. Therefore, activating apoptotic machinery in cancer cells using non-toxic phytochemicals could be an effective measure in PCA management. Phytochemical treatments could cause apoptotic death either by engagement of death receptors or by stimulation of intrinsic pathway in cancer cells.

Recent studies have demonstrated that Akt, NF- κ B and AR based signaling are the key regulator of cellular survival and apoptotic machinery; and uncontrolled activity of these signaling molecules is critical for the growth and progression of PCA [15,19–21]. Together, this axis constitutes a potential therapeutic target for the treatment of PCA. Therefore, in the present study, we analyzed the mechanistic details underlying the pro-apoptotic effect of isosilybin A and also analyzed its effect on the Akt, NF- κ B and AR signaling. Our results suggest that isosilybin A treatment activates apoptotic pathways in PCA cells via targeting the Akt-NF- κ B-AR axis.

MATERIALS AND METHODS

Reagents

Isosilybin A was isolated (purity >97%) from powdered extract (1 kg; lot 37501) of the fruits of *Silybum marianum* (L.) Gaertn. [obtained from Euromed, S.A. (Barcelona, Spain), a part of the Madaus Group (Köln, Germany)] as described in detail previously [22]. Antibodies for cleaved Poly (ADP-ribose) polymerase (cPARP), cleaved caspase 3, cleaved caspase 8, cleaved caspase 9, phospho-Akt (Serine-473), total Akt, phospho-Bcl2 (Serine-70), total Bcl2, BAD, BAX, BID, SMAC/DIABLO, and anti-rabbit peroxidase-conjugated secondary antibody were obtained from Cell Signaling (Beverly, MA, USA). Antibodies for DR5, p65, p50 and AR were from Santa Cruz Biotechnology (Santa Cruz, CA). PSA antibody was from Dako A/S, Denmark and antibody for β -actin was from Sigma-Aldrich Chemical Co. (St Louis, MO). α -Tubulin antibody was from Neomarkers, Fremont, CA. ECL detection system and anti-mouse HRP-conjugated secondary antibody were from GE Healthcare (Buckinghamshire, UK). RPMI1640 media, Iscoves's modified Dulbecco's minimal essential medium (IMDM), fetal bovine serum (FBS) and penicillin-streptomycin (P-S) (10,000 units/ml penicillin and 10,000 μ g/ml streptomycin) were from Invitrogen Corporation (Gaithersburg, MD). Bio-Rad detergent-compatible protein assay kit was from Bio-Rad Laboratories (Hercules, CA). Pan-caspase inhibitor (Z-VAD.fmk) was obtained from Enzyme System Products (Livermore, CA). All other reagents were obtained in their highest purity grade available commercially.

Cell Culture

Human prostate cancer LNCaP and 22Rv1 cells were from the American Type Culture Collection (Manassas, VA). LAPC4 cells were kindly provided by Dr Robert E. Reiter (UCLA, CA, USA). LNCaP and 22Rv1 cells were grown in RPMI-1640 media supplemented with 10% FBS and 1% P-S at 37°C in a humidified 95% air and 5% CO₂ atmosphere. LAPC4 cells were cultured in Iscoves's modified Dulbecco's medium with 15% FBS, and 1% penicillin-streptomycin (P-S). Cells were treated with different concentrations (10–180 μ M) of isosilybin A in DMSO for desired durations, and total cell lysates or nuclear/cytoplasmic lysates were prepared as described earlier [23,24]. An equal amount of DMSO (vehicle) was present in each treatment including control, which did not exceed 0.1% (v/v). Cells were also pre-treated with Z-VAD.fmk (50 μ M for 2h) followed by desired isosilybin A treatment.

Apoptosis assay

Apoptosis assay was performed by the Hoechst assay as described previously [25]. Briefly, cells (22Rv1, LAPC4 or LNCaP) were plated to about 40–50% confluency and treated with isosilybin A (90–180 μ M). At the end of each treatment time (24 or 48h), both adherent and non-adherent cell populations were collected by brief trypsinization followed by washing with ice-cold PBS. Cells were then stained with DNA binding dye Hoechst 33342 and PI, and the apoptotic population was quantified using a fluorescent microscope (Zeiss, Axioskop 2 plus-HBO 100) by counting cells/microscopic field (at 400x) in five fields in each triplicate sample. Apoptotic dead cells showed orange-red fluorescence (Hoechst-stained), which was distinguished from necrotic cells showing bright red fluorescence (PI-stained).

Western Blotting

For western blotting, lysates (40–70 μ g) were denatured in 2X SDS-PAGE sample buffer and were resolved on 8–16% Tris-glycine gels. The separated proteins were transferred to nitrocellulose membrane followed by blocking with 5% non-fat milk powder (w/v) in Tris-

buffered saline (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20) for 1h at room temperature. After blocking, the membranes were probed with desired primary antibodies for 2 h at room temperature and then overnight at 4°C followed by appropriate peroxidase-conjugated secondary antibody for 1 h at room temperature and visualized by ECL detection system. In each case, blots were subjected to multiple exposures on the film to make sure that the band density is in the linear range. For all results autoradiograms/ bands were scanned with Adobe Photoshop 6.0 (Adobe Systems Inc., San Jose, CA). To ensure equal protein loading, each membrane was stripped and reprobed with β -actin antibody or α -tubulin antibody.

Statistics

Statistical analyses were performed using SigmaStat 2.03 software (Jandel Scientific, San Rafael, CA). Data were analyzed using one-way ANOVA followed by Bonferroni *t*-test, and a statistically significant difference was considered to be at $p < 0.05$.

RESULTS

Isosilybin A treatment induces apoptotic death in human PCA cells

Our earlier study provided the preliminary evidence that isosilybin A treatment induces apoptotic death in PCA cells [13], but its detailed effect on apoptosis remained unknown. In the present study, we examined the detailed effect of isosilybin A on apoptosis and cell survival related signaling molecules in three different human PCA cell lines carrying functional AR namely 22Rv1, LAPC4 and LNCaP.

First, we analyzed the effect of isosilybin A treatment on the induction of apoptotic death in PCA cells by Hoechst/PI assay. As shown in figure 1A, isosilybin A (90–180 μ M) increased the apoptotic population by 3.1–4.6 folds and 3.7–4.8 folds after 24 and 48h of treatment respectively in 22Rv1 cells. In LAPC4 cells, isosilybin A (90–180 μ M) caused 3–5.2 folds and 4.1–5.6 folds increase in apoptotic population after 24 and 48h of treatment respectively (Figure 1B). Similarly, in LNCaP cells, isosilybin A treatment (90–180 μ M) increased the apoptotic population by 2.7–4.0 folds and 3.1–4.9 folds after 24 and 48h of treatment respectively (Figure 1C). These studies showed that isosilybin A treatment induces apoptosis in different PCA cell lines.

Isosilybin A treatment activates extrinsic and intrinsic pathways of apoptosis

Next, we examined the effect of isosilybin A on various molecules involved in extrinsic and intrinsic pathways of apoptosis in above mentioned human PCA cells. Our results showed that isosilybin A treatment (90 and 180 μ M) activates both extrinsic and intrinsic pathways of apoptosis in 22Rv1, LAPC4 and LNCaP PCA cells. In 22Rv1 cells, isosilybin A treatment for 24 and 48h resulted in a strong increase in the level of cPARP, a known downstream substrate of caspase 3 and a well-known marker for apoptosis (Figure 2A). The increase in cPARP was associated with increased levels of DR5 and cleaved caspase 8 as well as increased levels of cleaved caspase 9 and 3 (Figure 2A). In 22Rv1 cells, isosilybin A treatment also decreased the phosphorylation of Bcl2 at the Serine-70 site along with a slight decrease in the total level of Bcl2; however, it did not significantly affect the levels of BAD, BAX, cleaved BID or SMAC/DIABLO under similar treatment conditions (data not shown). Similar to results in 22Rv1 cells, isosilybin A treatment increased the level of cPARP, DR5, cleaved caspase 8, cleaved caspase 9 and 3 in LAPC4 and LNCaP cells (Figure 2B and 2C). We also examined the effect of a range of isosilybin A concentrations (10–180 μ M) on cPARP and cleaved caspase 3 revealing that the effect of isosilybin A on these molecules is concentration-dependent (Figure 2D). Membranes were stripped and reprobed with α -tubulin antibody to confirm equal protein loading. As the aforementioned results showed a

strong activation of apoptotic machinery by isosilybin A, we next examined the effect of isosilybin A on key signaling molecules (Akt, NF- κ B and AR), which are known to directly or indirectly regulate apoptosis in PCA cells [15,19–21].

Isosilybin A treatment decreases phospho- and total Akt in PCA cells

Akt is a serine/threonine kinase that plays an important role in cell survival and inhibition of apoptosis [15,21]. The increased expression of Akt, either phosphorylated or non-phosphorylated form, has been associated with high Gleason scores as well as poor prognosis in PCA [26–28]. Our results showed that isosilybin A treatment reduces Akt phosphorylation at the Serine-473 site, which was more pronounced in LNCaP and LAPC4 cells compared to 22Rv1 cells (Figure 3A, B & C). The decrease in Akt phosphorylation by isosilybin A was accompanied with reduced total levels of Akt (Figure 3A, B & C). Our results also showed that even the 10 μ M dose of isosilybin A was effective in decreasing the phosphorylation of Akt in LNCaP cells (Figure 3D). In all cases, membranes were stripped and reprobed with β -actin antibody to confirm equal protein loading.

Isosilybin A treatment decreases nuclear levels of p50 and p65

NF- κ B is a transcription factor that regulates the expression of genes involved in cell proliferation, anti-apoptosis, angiogenesis, invasion and metastasis of PCA cells [29–31]. In normal cells, NF- κ B is localized in the cytoplasm as an inactive complex bound by inhibitors known as I κ Bs (inhibitor of kappa B). Various signals can lead to phosphorylation and degradation of I κ Bs with the resultant translocation of active NF- κ B into the nucleus, where it binds to specific DNA sites and regulates gene expression. Next, we examined the effect of isosilybin A treatment on two important members of the NF- κ B family (p50 and p65). Results showed that isosilybin A treatment decreases the level of p50 and p65 in the nuclear fraction of 22Rv1, LAPC4 and LNCaP cells after 24 and 48h of treatment (Figure 4A, B & C). The effect of isosilybin A was more pronounced on p50 levels compared to that of p65, and was prominent mainly at the 180 μ M concentration (Figure 4A, B & C). Under similar treatment conditions, isosilybin A had no significant effect on the levels of p50 and p65 in the cytoplasmic fractions (Figure 4A, B & C). Purity of nuclear and cytoplasmic fractions was confirmed by re-probing membranes with histone H1 and tubulin, detectable only in nuclear and cytoplasmic fractions, respectively.

Isosilybin A treatment decreases AR and PSA level in PCA cells

AR is a ligand-dependent transcription factor whose activation is initiated by androgen binding and its subsequent translocation to the nucleus. There, it binds to the DNA as a homodimer on androgen responsive elements (AREs) within the regulatory regions of target genes and regulates transcription of AR target genes such as prostate-specific antigen (PSA) [32–34]. PSA is a kallikrein-like serine protease that serves as an important clinical biomarker to monitor diagnosis, treatment response, prognosis, and progression in PCA patients [35]. Next, we examined the effect of isosilybin A treatment on AR and PSA level in PCA cells, where it decreased the total AR level in 22Rv1, LAPC4 and LNCaP cells (Figure 5A, B & C). We also observed a strong decrease in the levels of PSA (both secreted as well as intracellular) in all the three PCA cell lines. Further, our results in LNCaP cells also showed that the inhibitory effect of isosilybin A treatment (10–180 μ M) on PSA level was dose-dependent (Figure 5D). In all cases, membranes were stripped and reprobed with β -actin antibody to confirm equal protein loading.

Role of caspases in Isosilybin A-mediated decrease in the AR

Next, we examined whether the isosilybin A-mediated decrease in the AR was due to activation of caspases. To accomplish that we pre-treated the 22Rv1, LNCaP and LAPC4

cells with pan-caspase inhibitor (Z-VAD.fmk) (50 μ M for 2h) followed by isosilybin A (180 μ M) treatment for 24h, and then examined the levels of AR, cPARP and caspases. Results showed that in the presence of pan-caspase inhibitor, isosilybin A-mediated AR decrease was largely compromised in 22Rv1 cells, but had only marginal effect in LAPC4 and LNCaP cells (Figure 6A). To further delineate the primary target of isosilybin A, next, we treated 22Rv1 cells with isosilybin A (180 μ M) and conducted a temporal kinetic analysis of AR, cPARP and cleaved caspase 3. As shown in figure 6B, isosilybin A treatment resulted in significant AR decreases starting at 4h, while cPARP and caspase 3 activation were evident only after 12h of Isosilybin A treatment. These results are suggestive that the effect of Isosilybin A on AR could be a primary event as these decreases were evident much earlier relative to caspase activation and apoptosis induction (Figure 6B).

Discussion

PCA remains a leading cause of cancer related incidences and deaths among men in the United States [1]. To lower the rising PCA burden, more efforts are sought for developing selective and biological mechanism-based therapies that are effective against PCA cells. In this regard, considerable attention has been focused on identifying naturally occurring non-toxic chemopreventive or chemotherapeutic phytochemicals with the aim of inhibiting, retarding or reversing the carcinogenesis process leading to PCA [2–4]. In this context, the results from the present study are noteworthy as isosilybin A treatment inhibited cell survival signaling and activated intrinsic and extrinsic pathways of the apoptotic machinery in different PCA cells.

Death receptor 5 (DR5) belongs to the tumor necrosis factor receptor (TNFR) gene superfamily and is considered an attractive therapeutic target to induce apoptosis in a variety of cancers [17,18,36]. DR5 contains an extracellular ligand-binding domain and a homologous cytoplasmic death domain (DD) that mediate its interaction with DD-containing adaptor proteins such as FADD (Fas-associated death domain) or TRADD (TNF-associated death domain) [17,18]. These adaptor molecules regulate the activation of downstream signaling molecules including caspase 8. The activated caspase 8 could activate caspase 3 either directly or via mitochondrial engagement through cleavage of BID and activation of caspase 9 [16–18]. In our study, isosilybin A treatment did not result in the cleavage of BID (data not shown); therefore, it could be presumed that the isosilybin A-mediated activation of caspase 9 is independent of caspase 8 activation. It has also been reported earlier that the cellular DNA damage could induce transcriptional upregulation of DR5 through p53 dependent and independent mechanisms [37]. In our previously published work, we have reported that isosilybin A treatment increases the cellular pool of total p53. Whether that is responsible for isosilybin A-mediated increased levels of DR5 remains to be studied in future studies.

Activated Akt is known to control crucial tumor-associated cellular processes, including cell proliferation, cell cycle progression, tissue invasion, migration, and suppression of anoikis and apoptosis [15,38,39]. Several studies have also reported that inhibition of the PI3K/Akt pathway leads to sensitization of cancer cells to apoptosis [15,38–40]. The anti-apoptotic role of Akt is mediated through phosphorylation and inactivation of various apoptosis related proteins like caspase 9 and BAD [41]. In the present study, we observed a strong decrease in Akt phosphorylation by isosilybin A treatment, which could directly or indirectly contribute to the apoptosis induction in PCA cells.

The transcription factor NF- κ B plays an important role in tumor growth, progression and chemotherapy resistance through its ability to suppress cell death pathways [30,31,42]. Regulation by NF- κ B has been described for more than 150 genes including many anti-

apoptotic proteins such as Bcl2, Bcl-xL and various IAPs (Inhibitor of apoptosis proteins) [43]. Webster et al. reported that NF- κ B could abrogate p53 mediated apoptosis by competing for nuclear co-activators such as p300/CBP [44]. Results from present study indicate that isosilybin A treatment decreases the nuclear levels of p50 and p65 in three PCA cell lines, which could result in a decreased expression of various anti-apoptotic proteins. Therefore, isosilybin A-mediated decreases in the nuclear level of NF- κ B members could directly potentiate the cellular apoptotic machinery.

The abnormal modulation of the androgen-AR signaling cascade plays a critical role in the growth and progression of human PCA [45,46]. Androgen deprivation has been reported to induce apoptosis in PCA cells, which could be overcome by AR overexpression during progression towards hormone-refractory stage [47–49], demonstrating the anti-apoptotic and pro-survival role of AR in PCA cells. A study by Liao et al. suggested a direct role of AR in apoptosis regulation in PCA cells [50]. This study illustrated that the silencing of AR using small-interfering RNA leads to apoptotic death in PCA cells [50]. Furthermore, this study also demonstrated the involvement of AR in the regulation of Bcl-xL expression, which is an important anti-apoptotic member of the Bcl2 family [50]. Results from the present study showed an inhibitory effect of isosilybin A on AR signaling, which could result in decreased survival as well as activation of apoptotic machinery in PCA cells. Importantly, our results also showed that effect of Isosilybin A on AR could be a primary event as a decrease in AR was evident much earlier relative to caspase activation and apoptosis induction.

As mentioned earlier, the Akt-NF- κ B-AR signaling complex plays a critical role in the progression of PCA [15,19–21]. Recent studies have shown a substantial degree of cross-talk among these three molecules [12,15,19,20,51–53]. The role of Akt has been established in the activation of NF- κ B via phosphorylation through activation of IKK [15]. Further, Akt has been reported to phosphorylate AR at specific sites leading to its activation or degradation depending upon the cellular physiological context or presence/absence of other signaling molecules [12,19–21]. Similarly, the relationship between NF- κ B and AR remained controversial. NF- κ B binding sites have been reported in the promoter region of AR, and NF- κ B has been shown to play a role in AR activation under various conditions [52]. A role for NF- κ B has also been reported in the activation of PSA through direct binding to a transcriptional enhancer in the PSA gene [53]. Contrary to these results, Palvimo et al. reported that the elevated expression of NF- κ B represses AR-mediated transactivation [51]. This complex interaction between these three molecules is suggestive of the fact that a simultaneous targeting of the Akt-NF- κ B-AR signaling axis is indispensable for an effective treatment of clinical PCA. Therefore, the results from present study are highly significant as isosilybin A strongly inhibits all the three members of the complex Akt-NF- κ B-AR signaling axis.

In conclusion, the present study showed that isosilybin A could activate extrinsic and intrinsic pathways of apoptosis through targeting of the Akt-NF- κ B-AR axis. Since targeting apoptosis is a promising strategy for cancer treatment and management, Isosilybin A could be an important addition to the arsenal of selective non-toxic drugs in our fight against cancer.

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Abbreviations

PCA	Prostate cancer
PSA	Prostate specific antigen
AR	Androgen receptor
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
DR5	death receptor 5
Akt	Protein kinase B
NF-κB	Nuclear Factor kappa B cells
TNF	Tumor necrosis factor
IκB	Inhibitor of kappa B
IKK	Inhibitor of kappa B kinase
APAF1	apoptotic protease-activating factor 1
DMSO	Dimethyl Sulfoxide
FADD	Fas-associated death domain
IAPs	Inhibitor of apoptosis proteins
ANOVA	analysis of variance

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Figure 1. Isosilybin A induces apoptosis in human PCA 22Rv1, LAPC4 and LNCaP cells
 (A–C) 22Rv1, LAPC4 and LNCaP cells were treated at 40–50% confluency with DMSO or isosilybin A (90, 135 and 180 μ M) for 24 and 48h. At each treatment time, both adherent and non-adherent cell populations were collected and stained with DNA binding dyes Hoechst 33342/PI and analyzed for apoptotic population as detailed in the ‘Materials and Methods’. The quantified apoptosis data is presented as ‘fold change’ and each bar represents mean $\pm$ standard error of mean of three samples. *, $p < 0.001$; Iso A, isosilybin A.

Figure 2. Effect of Isosilybin A on various members of apoptotic machinery in human PCA 22Rv1, LAPC4 and LNCaP cells

(A–C) 22Rv1, LAPC4 and LNCaP cells were treated at 40–50% confluency with DMSO or isosilybin A (90 and 180 μ M) for 24 and 48h. At each treatment time, both adherent and non-adherent cells were harvested and cell lysates were prepared in non-denaturing lysis buffer. For each sample, 60–75 μ g of protein lysate was used for SDS-PAGE and Western immunoblotting, and membranes were probed for cleaved PARP, DR5, cleaved caspase 8, cleaved caspase 9, cleaved caspase 3, phosphorylated and total Bcl2. (D) LNCaP cells were treated with DMSO or isosilybin A (10–180 μ M) for 48h and total cell lysates were analyzed for cPARP and cleaved caspase 3 by Western blotting. Membranes were also stripped and reprobed with anti-tubulin antibody to confirm equal protein loading.

Figure 3. Effect of Isosilybin A on phospho- and total Akt levels in human PCA 22Rv1, LAPC4 and LNCaP cells

(A–C) 22Rv1, LAPC4 and LNCaP cells were treated at 40–50% confluency with DMSO or isosilybin A (90 and 180 μ M) for 24 and 48h. At each treatment time, both adherent and non-adherent cells were harvested and cell lysates were prepared in non-denaturing lysis buffer. For each sample, 50–70 μ g of protein lysate was used for SDS-PAGE and Western immunoblotting, and membranes were probed for phospho-Akt Serine-473 and total Akt.

(D) LNCaP cells were treated at 40–50% confluency with DMSO or isosilybin A (10–180 μ M) for 48h and total cell lysates were analyzed for phospho-Akt Serine-473 and total Akt by Western blotting. In each case, protein loading was checked by stripping and re-probing the membranes with β -actin antibody.

Figure 4. Effect of Isosilybin A on p50 and p65 level in the cytoplasmic and nuclear fractions of human PCA 22Rv1, LAPC4 and LNCaP cells

(A–C) 22Rv1, LAPC4 and LNCaP cells were treated at 40–50% confluency with DMSO or isosilybin A (90 and 180 μ M) for 24 and 48h. At indicated treatment times, both adherent and non-adherent cells were harvested and nuclear/cytoplasmic fractions were prepared and Western blotting was carried out for p50 and p65. Purity of nuclear and cytoplasmic fractions was confirmed by re-probing membranes with histone H1 and tubulin, detectable only in nuclear and cytoplasmic fractions, respectively.

Figure 5. Effect of Isosilybin A on AR and PSA levels in human PCA 22Rv1, LAPC4 and LNCaP cells

(A–C) 22Rv1, LAPC4 and LNCaP cells were treated at 40–50% confluency with DMSO or isosilybin A (90 and 180 μ M) for 24 and 48h. At each treatment time, media was collected. Further, both adherent and non-adherent cells were harvested and cell lysates were prepared in nondenaturing lysis buffer. For each sample, 40–60 μ g of protein lysate was used for SDS-PAGE and Western immunoblotting, and membranes were probed for AR and PSA (intracellular and secreted). (D) LNCaP cells were treated at 40–50% confluency with DMSO or isosilybin A (10–180 μ M) for 48h. Thereafter, media and total cell lysates were analyzed for PSA by Western blotting. Protein loading was checked by stripping and re-probing the membranes with β -actin antibody. For secreted PSA, loading volume of media was normalized with respective protein values of the total cell lysates.

Figure 6.

(A) Effect of Isosilybin A-mediated caspases activation on AR level in human PCA 22Rv1, LAPC4 and LNCaP cells. 22Rv1, LAPC4 and LNCaP cells were pre-treated with pan-caspase inhibitor Z-VAD.fmk (50 μ mol/L, 2h), and incubated with DMSO or isosilybin A (180 μ M) for another 24h. At the end of the treatment, both adherent and non-adherent cells were harvested and cell lysates were prepared in nondenaturing lysis buffer. For each sample, 50–75 μ g of protein lysate was used for SDS-PAGE and Western immunoblotting, and membranes were probed for AR, cPARP, cleaved caspase 9 and cleaved caspase 3. Protein loading was checked by stripping and re-probing the membranes with tubulin antibody. **(B) Temporal kinetic analysis of Isosilybin A effect on AR, cPARP and cleaved caspase 3.** 22Rv1 cells were treated with DMSO or isosilybin A (180 μ M) and cell lysates were prepared after 4, 8, 12, 18 and 24h of treatment. For each sample, 50–70 μ g of protein lysate was used for SDS-PAGE and Western immunoblotting, and membranes were probed for AR, cPARP and cleaved caspase 3. Membranes were stripped and re-probed with β -actin antibody to confirm equal protein loading. Iso A, isosilybin A; CI, caspase inhibitor.