The hedgehog pathway inhibitor cyclopamine increases levels of p27, and decreases both expression of IGF-II and activation of Akt in PC-3 prostate cancer cells ☆

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Abstract

The hedgehog signalling inhibitor cyclopamine has been shown to induce growth inhibition and cell cycle arrest in prostate cancer cell lines, but the mechanism of action has not been clearly defined, and observations between laboratories have not always been consistent. We first observed that albumin can protect PC-3 prostate cancer cells from cyclopamine-induced growth inhibition, suggesting that cyclopamine binds to albumin, and that only free cyclopamine is active. We then conducted a phospho-site protein kinase screen to elucidate the mechanism of cyclopamine-induced growth inhibition. Treatment of PC-3 cells with 5 or 10 μM cyclopamine for 72 h resulted in a decrease in cell viability of ~50% and ~75%, respectively. A phospho-site protein kinase screen showed that cyclopamine decreased levels of phospho-Thr^{187}-p27 by 71%. This phospho-site on p27 positively regulates its ubiquitin degradation; therefore a decrease in phospho-Thr^{187}-p27 should correlate with increased levels of p27. Consistent with this hypothesis, treatment of PC-3 cells with cyclopamine resulted in a ~3-fold increase in p27 protein levels. Cdk-2 phosphorylates Thr^{187}-p27, and immunoblotting demonstrated that cyclopamine treatment of PC-3 cells reduces the expression of cdk-2. Furthermore, cyclopamine decreased the levels of phosphorylated (activated) Akt, which is known to increase p27 degradation via Skp-2-induced ubiquitination. The mechanism by which cyclopamine decreases phosphorylated Akt is currently under investigation, but it may involve our observed cyclopamine-induced reduction in IRS-1 and IGF-II expression. These results demonstrate novel molecular correlates of cyclopamine-induced growth inhibition of prostate cancer cells.

Keywords: Cyclopamine; Hedgehog; Insulin-like growth factor; Akt; p27

1. Introduction

The hedgehog signalling pathway is an important regulator of embryonic development (for a review, see Ref. [1]). Three ligands (sonic hedgehog, Indian hedgehog, and desert hedgehog) bind

to the 12-pass transmembrane protein patched (PTCH), which functions as a repressor of the pathway. Hedgehog binding to PTCH relieves its inhibition of smoothened (SMOH), which is a 7-pass transmembrane responsible for transducing the hedgehog signalling pathway. Activation of SMOH leads to upregulation of the GLI family of transcription factors, and subsequent downstream transcriptional targets, which include TGF-β, cyclins, and BMP-2 [1]. Cyclopamine is a steroidal alkaloid which inhibits the hedgehog signalling pathway by directly interacting with SMOH [2].

Recent publications have demonstrated a positive correlation between hedgehog signalling and prostate cancer progression [3–5]. This suggests a potential use of cyclopamine or analogues for the treatment of prostate cancer, but the mechanism of action of cyclopamine has not yet been clearly defined. Cyclopamine has been shown to induce growth inhibition in several prostate cancer cell lines *in vitro* [3–5], and we wished to investigate the downstream modulators of this effect. However, some discrepancies exist in prior reports. For example, Karhadkar et al. have shown that DU145 cells are significantly growth inhibited by 1–3 μM cyclopamine [3], whereas Sanchez et al. have reported that 10 μM cyclopamine had no effect on the proliferation of this cell line [4]. Furthermore, Sanchez et al. have reported that LNCaP cells are extremely sensitive to cyclopamine-induced growth inhibition [4], whereas Fan et al. did not observe any growth inhibition of LNCaP with cyclopamine [5]. However, both the Karhadkar and Sanchez study demonstrated that PC-3 cells respond to the growth inhibitory actions of cyclopamine [3,4]. Since PC-3 cells consistently respond to cyclopamine treatment, we chose to perform our study in this cell line. Furthermore, PC-3 cells are a model of androgen-independent prostate cancer, a disease in which current treatment modalities are not effective [6].

2. Materials and methods

2.1. Cell culture

The human androgen-independent prostate cancer cell line PC-3 was obtained from the American Type Culture Collection (Manassas, VA). The cells were maintained in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS) at 37 °C and 5% CO_2. Cyclopamine was obtained from Toronto Research Chemicals (Toronto, ON, Canada).

2.2. Cell proliferation assay

We used a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay to determine cell proliferation (cell viability). Cells (1.0×10^5/well) were plated in six-well plates in regular growth media. After the appropriate treatment time (as indicated in figure legends), MTT (Sigma Chemical Co., St. Louis, MO) was added to a final concentration of 1 mg/ml, the reaction mixture was incubated for 3 h at 37 °C. The resulting crystals were dissolved in 0.04% HCl in isopropanol and the absorbance was read at 562 nm. The data are presented in bar graphs as means plus or minus standard error of the mean. Each experiment was performed in triplicate. To assess the statistical significance of observed differences, we used Student's *t*-test. All tests were two-sided, and *P* values less than 0.05 were considered to be statistically significant.

2.3. Western blotting

After each treatment, whole cell lysates were obtained using RIPA buffer (0.1 mM dibasic sodium phosphate, 1.7 mM monobasic phosphate, 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, and apoprotein at 0.2 U/ml). Quantitation of total protein concentrations in the cell lysates was determined by Bradford assay (Bio-Rad, Hercules, CA). Fifty micrograms of protein from cell lysates was resolved electrophoretically on denaturing 10–12% SDS–polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were probed with antibodies specific for p27, cdk-2 (Santa Cruz Biotechnology, Santa Cruz, CA), IRS-1 (Upstate, Lake Placid, NY), phospho-Ser^{473}-Akt (P-Akt), total Akt, and phospho-p70S6kinase (New England Biolabs, Beverly, MA). The position of protein was visualized with horseradish peroxidase-conjugated secondary antibodies (Amersham, Piscataway, NJ). To confirm equal loading, membranes were stripped and re-probed using an antibody specific for α-tubulin (Santa Cruz, San Diego, CA). In order to quantify the fold change in p27 protein levels, densitometry readings were performed using Scion Image software (Scion Corporation, Frederick, MD).

2.4. Phospho-site kinase screen

Cell lysates were obtained as described above. Hundred milligrams of total proteins were subjected to phosphorylation analysis on specific phosphorylation sites using Kinexus KPSS-10.0 phospho-site profiling screen software (Kinexus, Vancouver, BC, Canada). This analysis combines proprietary methodologies with the analytical techniques involving gel electrophoresis, immunoblotting, and protein visualization via enhanced chemi-

luminescence (ECL). Briefly, the KPSS-10.0 screen detects the phospho-sites in two steps. First, molecules were separated by gel electrophoresis based on their molecular weights, and then were detected by their immunoreactivity with highly validated phospho-specific antibodies. The resulting immune complexes were subjected to a multiplexing apparatus, and the quantitation of the bands is visualized using ECL followed by a highly sensitive imaging system with a 16-bit camera and a quantitation software to analyze the chemiluminescent samples. Each sample's immunoblot was scanned at its maximum time to ensure that the signal from the strongest immunoreactive protein on the immunoblot remains below saturation. It thus provides accurate quantitation over a 2000-fold range of linearity.

2.5. IGF-II quantitative real-time PCR (TaqMan)

Total cellular RNA was isolated from cells by the RNAzol-B method (Tel-Test, Friendswood, TX), according to the manufacturer's protocol. Two micrograms of total RNA was treated with DNAse I (Invitrogen) to remove DNA contamination. Then, the RNA was reverse-transcribed using MMLV reverse transcriptase (Invitrogen) in a 50-μl reaction mixture. TaqMan primers and probes for quantitative detection of IGF-II and 18S rRNA were designed with Primer Express (ABI-Perkin-Elmer) using the GenBank Accession Nos. (X03562 for IGF-II and M10098 for 18S). PCR primer and probe sequences were as follows (all 5′ to 3′ direction): IGF-II: forward GACCGCGGCTTCTACTTC, reverse ACGT CCCTCTCGGACTTG, probe: TGTTTCCGCAGCTG TGACCTG, for an amplicon length of 143 bp; 18S: forward CGGCTACCACATCCAAGGAA, reverse GCT GGAATTACCGCGGCT, probe: TGCTGGCACCAG ACTTGCCCTC, for an amplicon length of 188 bp. cDNA samples were mixed with 2× Universal TaqMan buffer containing the Taq enzyme, primers at a final concentration of 300 nM for IGF-II and 40 nM for 18S, and probe at a final concentration of 200 nM for IGF-II and 150 nM for 18S in a total volume of 25 μl. The thermal cycler conditions were 50 °C 2 min, 95 °C 10 min and 42 cycles of 95 °C 15 s and 60 °C 1 min. All PCRs and analysis were performed by using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). All samples were run in triplicate. The experiment was repeated twice.

2.6. IGF-II ELISA

After 72 h treatment in serum-free media, PC-3 conditioned media was collected and concentrated 40-fold using Centricons (Millipore, Bedford, MA). Concentrated conditioned media were subjected to IGF-II ELISA (DSL-10-9100, Diagnostic Systems Laboratories, Inc., Webster, TX), as per the manufacturer's protocol.

3. Results and discussion

Cyclopamine, an inhibitor of hedgehog signalling [2], has been previously shown to act as a growth inhibitor for various prostate cancer cell lines *in vitro* [3–5]. In order to confirm the ability of cyclopamine to inhibit the proliferation of PC-3 prostate cancer cells, our first set of experiments were designed to establish dose–response curves of PC-3 cells to cyclopamine. As seen in Fig. 1, treatment of PC-3 cells with 5 or 10 μM cyclopamine for 72 h resulted in a decrease in cell viability of ~50% and ~75%, respectively. These experiments were performed in low (0.5%) FBS-containing media, as higher FBS concentrations (10%) resulted in complete protection from the growth inhibitory effects of cyclopamine (data not shown).

We hypothesized that the protective effects of FBS could be due to the presence of growth factors such as insulin-like growth factor (IGF)-I and epidermal growth factor (EGF). We had previously determined that FBS-induced protection of breast cancer cells from herceptin

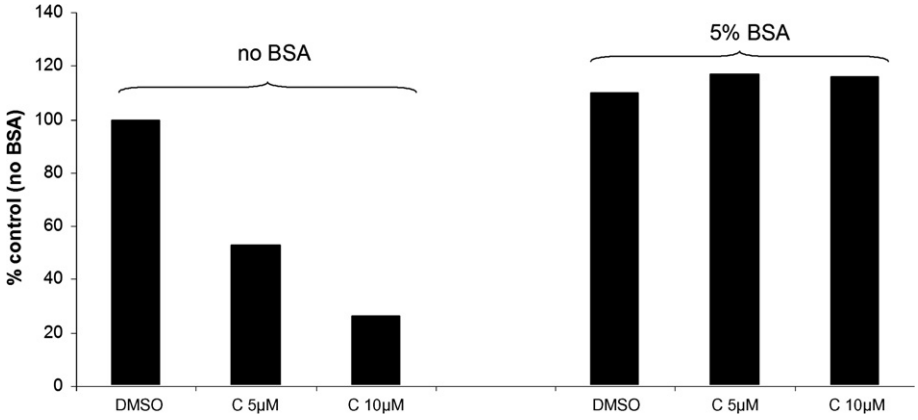
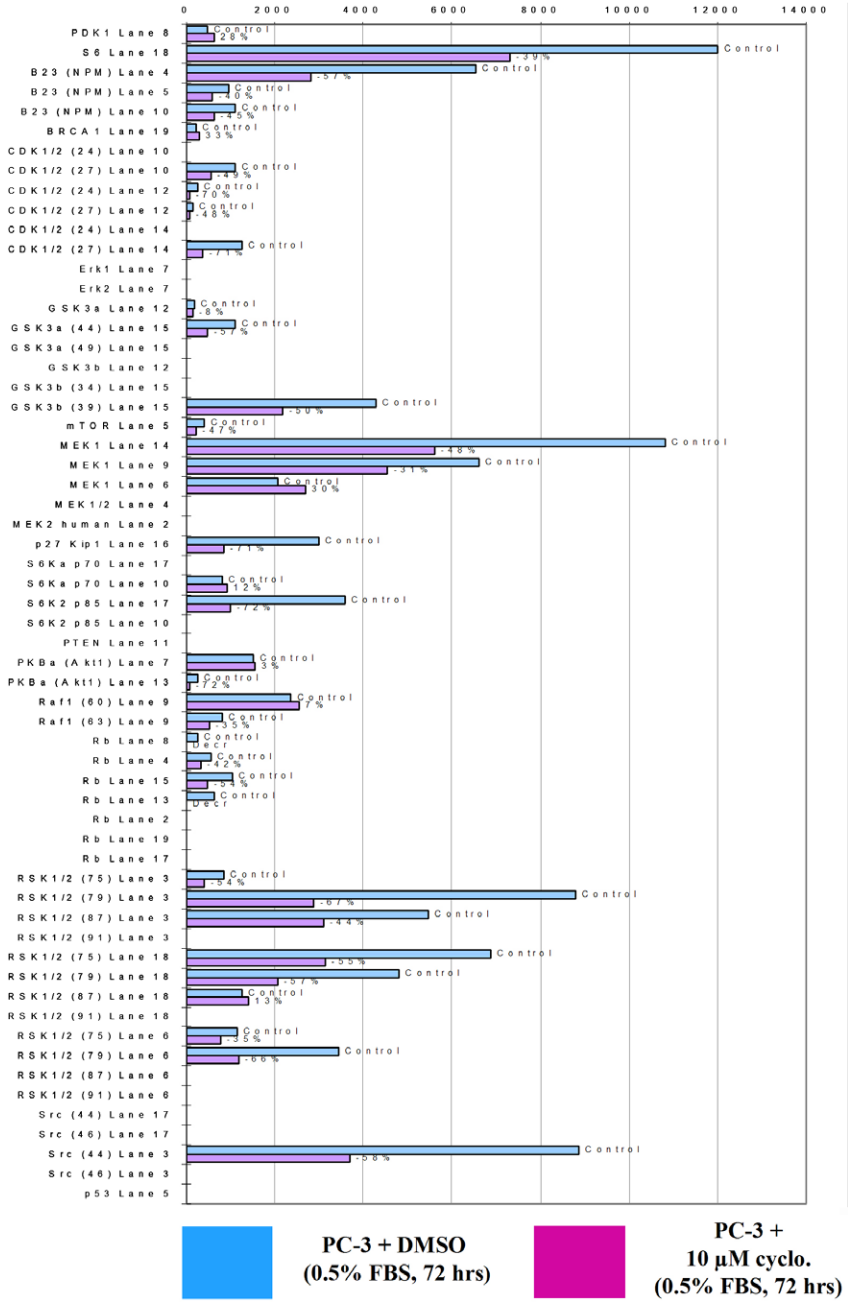


Fig. 1. Cyclopamine inhibits PC-3 proliferation. PC-3 cells were treated with DMSO (control), 5 μM cyclopamine (C 5 μM), or 10 μM cyclopamine (C 10 μM) for 72 h in RPMI/0.5% FBS and then MTT assay was performed.

[7], and of pancreatic cancer cells from celecoxib [8], was due to the presence of IGF-I in FBS. However, we did not observe any rescue of PC-3 cells from cyclopamine-induced growth inhibition in low FBS-containing media with the addition of either IGF-I (50 ng/ml), IGF-II (100 ng/ml), or EGF (50 ng/ml) (data not shown). We therefore hypothesized that a protein in FBS, such as bovine serum albumin (BSA), binds with cyclopamine and prevents it from acting as a growth inhibitor. As seen in Fig. 1, addition of 5% BSA to 0.5% FBS-containing media resulted in complete protection from cyclopamine-induced growth inhibition. This suggests that the



PDK1	S244
S6	S235
B23 (NPM)	S4
B23 (NPM)	T199
B23 (NPM)	T234+T237
BRCA1	S1497
CDK1/2	T14+Y15
CDK1/2	T14+Y15
CDK1/2	T161/T160
CDK1/2	T161/T160
CDK1/2	Y15
CDK1/2	Y15
Erk1	T202+Y204
Erk2	T185+Y187
GSK3a	S21
GSK3a	Y279
GSK3a	Y279
GSK3b	S9
GSK3b	Y216
GSK3b	Y216
mTOR	S2448
MEK1	S297
MEK1	T291
MEK1	T385
MEK1/2	S217/S221
MEK2 human	T394
p27 Kip1	T187
S6Ka p70	T229
S6Ka p70	T421/S424
S6K2 p85	T252
S6K2 p85	T444/S447
PTEN	S380+T382+S385
PKBa (Akt1)	S473
PKBa (Akt1)	T308
Raf1	S259
Raf1	S259
Rb	S612
Rb	S780
Rb	S807
Rb	S807+S811
Rb	T356
Rb	T821
Rb	T826
RSK1/2	S221/S227
RSK1/2	S221/S227
RSK1/2	S221/S227
RSK1/2	S221/S227
RSK1/2	S363/S369
RSK1/2	S363/S369
RSK1/2	S363/S369
RSK1/2	S363/S369
RSK1/2	S380/S386
RSK1/2	S380/S386
RSK1/2	S380/S386
RSK1/2	S380/S386
Src	Y418
Src	Y418
Src	Y529
Src	Y529
p53	S392

Fig. 2. Phospho-site protein kinase screen on PC-3 cells treated with 10 µM cyclopamine for 72 h. PC-3 cells were treated with DMSO (blue bars) or 10 µM cyclopamine (purple bars) in RPMI/0.5% FBS. After 72 h, whole cell lysates were collected, and sent for phospho-site kinase screen analysis (Kinexus, Vancouver, BC, Canada). (For interpretation of the references in color in this figure legend, the reader is referred to the web version of this article.)

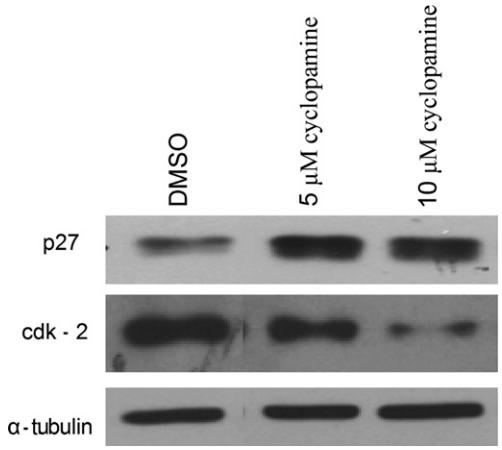


Fig. 3. Cyclopamine modulates the expression of p27 and cdk-2 involved in cell cycle progression and apoptosis. PC-3 cells were treated with DMSO (control) or various concentrations of cyclopamine (as indicated) in RPMI/0.5% FBS. After 72 h, whole cell lysates were collected and separated by SDS–PAGE. Western blots were performed for p27 and cdk-2, and α-tubulin levels were used as loading controls. Densitometry readings for p27 levels indicated a ~3-fold increase with either 5 μM or 10 μM cyclopamine treatment.

protective effects of FBS on cyclopamine-induced growth inhibition are due to direct interactions between the drug and BSA present in FBS.

In order to study the mechanism of cyclopamine-induced growth inhibition, lysates from cells treated for 72 h with 10 μM cyclopamine in 0.5% FBS-containing media were assayed using a phospho-site protein kinase screen. The array chosen included a number of genes involved in cell cycle regulation. The most striking result of the screen was that cyclopamine decreased the expression of phospho-Thr187-p27 by 71% (Fig. 2).

The cyclin-dependent kinase inhibitor p27 is known to induce cell cycle arrest and apoptosis in PC-3 cells [9]. The Thr^{187}-phospho-site on p27 induces the ubiquitin degradation of this protein [10,11]; therefore, a decrease in phospho-Thr187-p27 should correlate with increased levels of p27 protein due to decreased degradation. Consistent with this hypothesis, treatment of PC-3 cells with 5 or 10 μM cyclopamine resulted in a ~3-fold increase in p27 expression compared to DMSO treated cells, as assayed by Western blotting and densitometry (Fig. 3). The cell cycle regulator cdk-2 is a tyrosine kinase responsible for the phosphorylation of Thr187 on p27 [11,12], so we wished to verify if decreased cdk-2 expression or activity was responsible for our observed decrease in phospho-Thr187-p27 levels. Western blotting for cdk-2 levels in cell lysates from cyclopamine treated PC-3 cells revealed that cyclopamine reduces the expression of cdk-2 (Fig. 3). These results suggest that cyclopamine decreases cdk-2 expression, which subsequently results in decreased phospho-Thr187-p27, decreased p27 degradation, and increased p27 expression.

Cyclopamine also decreased the levels of phosphorylated (activated) Akt (Fig. 4), a known anti-apoptotic protein which plays an important role in cancer cell proliferation and survival [15]. The cyclopamine-induced decrease in P-Akt was more impressive when the cells were treated in serum-free media as opposed to 0.5% FBS-containing media (Fig. 4a vs. b). Activated Akt has

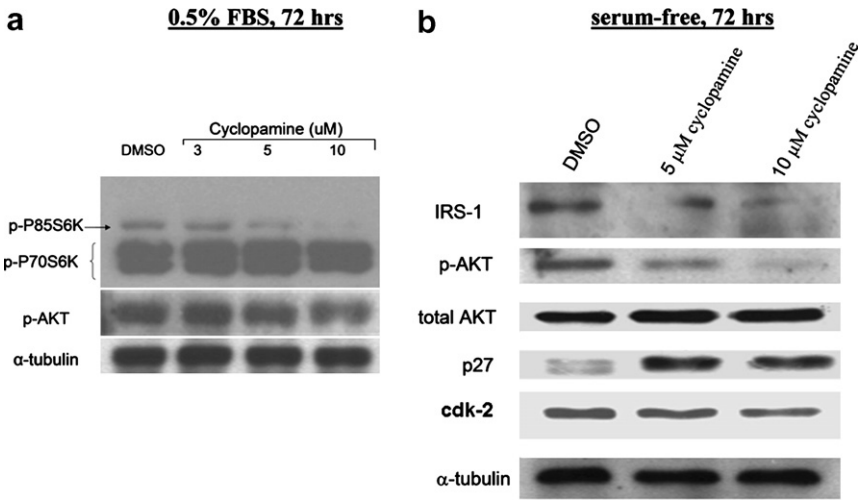


Fig. 4. Cyclopamine modulates the expression of genes involved in cell cycle progression and apoptosis. PC-3 cells were treated with DMSO (control) or various concentrations of cyclopamine (as indicated) in RPMI/0.5% FBS (a) or serum-free RPMI (b). After 72 h, whole cell lysates were collected and separated by SDS–PAGE. Western blots were performed for various proteins, and α-tubulin levels were used as loading controls.

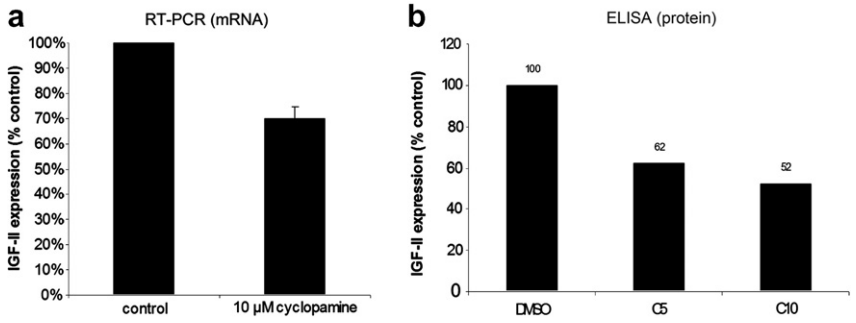


Fig. 5. Cyclopamine decreases the expression of IGF-II. PC-3 cells were treated with DMSO (control) or various concentrations of cyclopamine (as indicated) in serum-free RPMI. RNA was collected at 72 h and IGF-II mRNA expression was analyzed by quantitative real-time RT-PCR, as indicated in Section 2 (a). Conditioned media was collected at 72 h, and IGF-II protein expression was analyzed by ELISA, as indicated in Section 2 (b).

been shown to increase p27 degradation via Skp-2-induced ubiquitination in PC-3 cells [13]; therefore, cyclopamine may increase p27 protein expression by altering both cdk-2 expression and activation of Akt (Fig. 4). We also observed a decrease in the phosphorylation (activation) of P85S6K (Fig. 4a), a protein involved in translation which is activated by the downstream target of Akt, mTOR [14].

The mechanism by which cyclopamine inhibits Akt phosphorylation is currently under investigation, but one candidate mechanism is a decrease in the protein expression of IRS-1, as observed in Fig. 3b. IRS-1 is a mediator of insulin and IGF signalling, and is upstream of Akt in the signalling pathway [15]. We also hypothesized that the decrease in Akt activation may be due to a decrease in the expression of IGF-II, a ligand which activates the IGF-I receptor/IRS-1/PI-3 kinase/Akt pathway [15], and is expressed by PC-3 cells in an autocrine fashion [16]. This hypothesis was based on data from a study by Ingram et al., who used gene expression microarrays to demonstrate that one of the most substantial gene expression changes associated with transfection of SHH into pluripotent mesenchymal cells is an increase in IGF-II mRNA [17]. Therefore, it was plausible that the hedgehog inhibitor cyclopamine would result in decreased IGF-II levels. As seen in Fig. 5, cyclopamine decreased both the mRNA and protein expression of IGF-II in PC-3 cells (Fig. 5). Therefore, the observed decrease in P-Akt levels resulting from cyclopamine-treatment may be due to a decrease in the expression of IRS-1, IGF-II, or both. Interestingly, a recent paper has demonstrated that Akt is essential for hedgehog signalling [18]. Therefore, aside from the classic inhibitory action of cyclopamine on SMOH, cyclopamine-induced decrease in Akt activity may further contribute to inhibition of hedgehog signalling.

A recent publication has demonstrated no transcriptional activation of hedgehog target genes in response to hedgehog ligand or transfection with an activated form of SMOH in three prostate cancer cell lines (PC-3, LNCaP, and 22RV1) [19]. Furthermore, treatment of these cell lines with cyclopamine did not inhibit hedgehog target gene expression, although it inhibited proliferation *in vitro* [19]. We are currently trying to identify the novel targets(s) of cyclopamine responsible for our observed effects on the expression of p27, cdk-2, and P-Akt in PC-3 cells.

Our results define several novel molecular correlates of cyclopamine-induced growth inhibition of PC-3 prostate cancer cells. They draw attention to albumin-cyclopamine interactions as a variable that has a major impact on the *in vitro* growth inhibitory properties of this compound.

References

[1] K. Xie, J.L. Abbruzzese, Developmental biology informs cancer: the emerging role of the hedgehog signaling pathway in upper gastrointestinal cancers, Cancer Cell 4 (2003) 245–247.

[2] J.P. Incardona, W. Gaffield, R.P. Kapur, H. Roelink, The teratogenic Veratrum alkaloid cyclopamine inhibits sonic hedgehog signal transduction, Development 125 (1998) 3553–3562.

[3] S.S. Karhadkar, G.S. Bova, N. Abdallah, S. Dhara, D. Gardner, A. Mitra, J.T. Isaacs, D.M. Berman, P.A. Beachy, Hedgehog signalling in prostate regeneration, neoplasia and metastasis, Nature 431 (2004) 707–712.

[4] P. Sanchez, A.M. Herandez, B. Stecca, A.J. Kahler, A.M. DeGueme, A. Barette, M. Beyna, M.W. Datta, S. Datta, A.R.I. Altaba, Inhibition of prostate cancer proliferation by interference with SONIC HEDGEHOG-GLI1 signalling, Proc. Natl. Acad. Sci. USA 101 (2004) 12561–12566.

[5] L. Fan, C.V. Pepicelli, C.C. Dibble, W. Catbagan, J.L. Zarychi, R. Laciak, J. Gipp, A. Shaw, M.L.G. Lamm, A. Munoz, R. Lipinski, J.B. Thrasher, W. Bushman, Hedgehog signalling promotes prostate xenograph tumor growth, Endocrinology 145 (2004) 3961–3970.

[6] M. Gleave, H. Miyake, K. Chi, Beyond simple castration: targeting the molecular basis of treatment resistance in advanced prostate cancer, Cancer Chemother. Pharmacol. 56 (Suppl. 1) (2005) 47–57.

[7] Y. Lu, X. Zi, Y. Zhao, D. Mascarenhas, M. Pollak, Insulin-like growth factor-I receptor signaling and resistance to trastuzumab (Herceptin), J. Natl. Cancer Inst. 93 (2001) 1852–1857.
[8] R.J. Levitt, M. Pollak, Insulin-like growth factor-1 antagonizes the anti-proliferative effects of cyclooxygenase-2 inhibitors on BxPC-3 pancreatic cancer cells, Cancer Res. 62 (2002) 7372–7376.
[9] A.L. Katner, Q.B. Hoang, P. Gootam, E. Jaruga, Q. Ma, J. Gnarra, W. Rayford, Induction of cell cycle arrest and apoptosis in human prostate carcinoma cells by a recombinant adenovirus expressing p27(kip1), Prostate 53 (2002) 77–87.
[10] A. Montagnoli, F. Fiore, E. Eytan, A.C. Carrano, G.F. Draetta, A. Hershko, M. Pagano, Ubiquitination of p27 is regulated by Cdk-dependent phosphorylation and trimeric complex formation, Genes Dev. 13 (1999) 1181–1189.
[11] H. Nguyen, D.M. Gitig, A. Koff, Cell-free degradation of p27(kip1), a G1 cyclin-dependent kinase inhibitor, is dependent on CDK2 activity and the proteasome, Mol. Cell. Biol. 19 (1999) 1190–1201.
[12] R.J. Sheaff, M. Groudine, M. Gordon, J.M. Roberts, B.E. Clurman, Cyclin E-CDK2 is a regulator of p27(kip1), Genes Dev. 11 (1997) 1464–1478.
[13] P.W. van Duijn, J. Trapman, PI3K/Akt signaling regulates p27(kip1) expression via Skp2 in PC3 and DU145 prostate cancer cells, but is not a major factor in p27(kip1) regulation in LNCaP and PC346 cells, Prostate 66 (2006) 749–760.
[14] A.F. Shamji, P. Nghiem, S.L. Schreiber, Integration of growth factor and nutrient signaling: implications for cancer biology, Mol. Cell 12 (2003) 271–280.
[15] M.N. Pollak, E.S. Schernhammer, S.E. Hankinson, Insulin-like growth factors and neoplasia, Nat. Rev. Cancer 4 (2004) 505–518.
[16] H. Huynh, M. Pollak, J.C. Zhang, Regulation of insulin-like growth factor (IGF) II and IGF binding protein 3 autocrine loop in human PC-3 prostate cancer cells by vitamin D metabolite 1,25(OH)2D3 and its analog EB1089, Int. J. Oncol. 13 (1998) 137–143.
[17] W.J. Ingram, C.A. Wicking, S.M. Grimmond, A.R. Forrest, B.J. Wainwright, Novel genes regulated by sonic hedgehog in pluripotent mesenchymal cells, Oncogene 21 (2002) 8196–8205.
[18] N.A. Riobo, K. Lu, X. Ai, G.M. Haines, C.P. Emerson Jr., Phosphoinositide 3-kinase and Akt are essential for Sonic Hedgehog signaling, Proc. Natl. Acad. Sci. USA 103 (2006) 4505–4510.
[19] J. Zhang, R. Lipinski, A. Shaw, J. Gipp, W. Bushman, Lack of demonstrable autocrine hedgehog signaling in human prostate cancer cell lines, J. Urol. 177 (2007) 1179–1185.