Biochemical and Biophysical Research Communications 373 (2008) 109–112

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Autonomous Hedgehog signalling is undetectable in PC-3 prostate cancer cells

Frank R.K. McCarthy, Andrew J. Brown *

BABS, School of Biotechnology and Biomolecular Sciences, Biosciences Building D26, University of New South Wales, Sydney, NSW 2052, Australia

ARTICLE INFO

Article history:
Received 27 May 2008
Available online 9 June 2008

Keywords:
Hedgehog
PC-3 cells
Autocrine
Prostate cancer
Gli
Luciferase
Cyclopamine

ABSTRACT

The Hedgehog signalling pathway has been implicated in the development of prostate cancer, although this area remains controversial. Some but not all studies have noted relatively high Hedgehog pathway activity in commonly used prostate cancer cell lines. We aimed to evaluate the widely used PC-3 cell line as a model to investigate Hedgehog signalling in a prostate cancer setting. Using a sensitive Hedgehog inducible luciferase reporter assay, we found no evidence of autonomous Hedgehog signalling in PC-3 cells, irrespective of passage number. In addition, manipulations that should either increase (an oxysterol) or decrease (cyclopamine) Hedgehog pathway activity had no effect on reporter activity, and cyclopamine treatment did not affect PC-3 cell viability. Therefore, our findings contradict some earlier reports and caution against the use of PC-3 cells to investigate the Hedgehog pathway in a prostate cancer setting.

The Hedgehog (Hh) pathway plays a pivotal role in embryogenesis and patterning of vertebrate structures, including the prostate [1–3]. First described in *Drosophila melanogaster*, its three vertebrate orthologues are Sonic, Indian, and Desert Hh. The response to Hh is regulated by two transmembrane proteins, Patched and Smoothened. When Hh binds to its receptor, Patched, it relieves the Patched-inhibition of Smoothened. In turn, Smoothened, a distant relative of G protein-coupled receptors, induces downstream activation of the *gli* (glioma-associated) transcription factors involved in development. Cyclopamine, a teratogenic steroidal alkaloid, is commonly used to inhibit the Hh pathway by binding to Smoothened [4,5].

In addition to its role in foetal prostate development [1–3], several studies have implicated the Hh pathway in the development of prostate cancer, a significant cause of deaths in affluent nations. For instance, expression of Hh components and targets have been reported to be elevated in high-grade or metastatic prostate cancer [6–9]. In addition, cyclopamine treatment has been reported to decrease viability or proliferation of prostate cancer cells in culture [6–8], as well as in a xenograft mouse model [6]. Despite these studies, the involvement of the Hh pathway in prostate cancer is mired in controversy. There is ongoing debate regarding the autocrine *versus* paracrine nature of the signalling [2,10], and some studies have failed to find increased Hh signalling associated with prostate tumour development [11].

A major hurdle in advancing our understanding of the role of Hh in prostate cancer is the scarcity of suitable prostate cancer cell lines to study this pathway *in vitro*. Firstly, the difficulty of growing human prostate cancer cells *in vitro* has meant a lack of available cell lines to test [7]. Secondly, there are conflicting data for the most widely used prostate cancer cell lines, LnCaP, PC-3, and DU145. For example, in an influential study, Karhadkar et al. [6] found that these cell lines expressed more of a Hh target gene than a benign prostate epithelial cell line. In contrast, Zhang et al. [10] found that gene expression of Hh pathway components and targets was considerably lower in these cell lines than in pooled normal prostate tissue. In addition, Karhadkar et al. [6] used a Hh-responsive luciferase reporter assay to measure Hh pathway activity and found it to be elevated in these cell lines, since it could be abrogated by cyclopamine treatment [6]. Importantly, this evidence of an active Hh pathway was found in the absence of added Hh ligand, suggesting autocrine signalling, whereas Zhang et al. [10] were unable to demonstrate autocrine Hh signalling in these same human prostate cancer cell lines.

In the current study, we have employed the same Hh-responsive luciferase reporter assay used by Karhadkar et al. [6], with additional controls, to confirm or refute the presence of autocrine Hh signalling in a commonly used prostate cancer cell line. We have focused on PC-3 cells, an androgen insensitive cell line derived from a bone marrow metastasis, since this cell line appeared to display consistently higher Hh pathway activity than LnCaP or DU145 cells, across a number of studies and parameters [6–8]. We have complemented these experiments with studies using cyclopamine treatment and measurements of cell viability.

* Corresponding author. Fax: +61 2 9385 1483.
E-mail address: aj.brown@unsw.edu.au (A.J. Brown).

0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.05.169

Materials and methods

Materials. Commercial suppliers are listed in alphabetical order along with the materials purchased. Biomol Research Laboratories Inc. (PA, USA): tomatidine–HCL and cyclopamine. Invitrogen (Carlsbad, CA, USA): RPMI Medium 1640 + GlutaMAX-1 + 25 mM Hepes 1×, geneticin (G418), Lipofectamine 2000 transfection reagent, foetal bovine serum, OptiMEM I, penicillin–streptomycin. Nunc (Noble Park North, Vic): 24-well and 96-well cell culture plates. Promega: Dual-Luciferase reporter assay system, phRL-TK *Renilla* plasmid. Roche: Cell Proliferation Reagent water soluble tetrazolium salt (WST-1). R&D Systems: Culturex Poly-L-Lysine. Sigma–Aldrich (Castle Hill, NSW): Dulbecco's phosphate-buffered saline. Steraloids (Newport, RI, USA): 25-Hydroxycholesterol (cholest-5-en-3β, 25-diol). Lipoprotein-deficient calf serum was prepared as described [12]. The plasmids used were gifts from Dr. Hiroshi Sasaki (Center for Developmental Biology, RIKEN Kobe, Japan) [13]: 8×3′Gli-BSδ51LucII (8×GBS-luciferase where the GBS stands for Gli-binding sites), 8×m3′Gli-BSδ51LucII (8×mutGBS-luciferase) and pcDNA3.1-His-Gli1 (Gli-pcDNA).

Cell culture. PC-3 cells are derived from a secondary tumour formed in the bone marrow from a metastatic prostate cancer and are androgen independent [14,15]. Cells with higher passage numbers (50–54) were a gift from Professor Pamela Russell (Oncology Research Unit, The Prince of Wales Hospital, Sydney, Australia). PC-3 cells with a lower passage number (20–22), similar to that currently available from the American Type Culture Collection (passage number: 23 and 24), were a gift from Dr. Qihan Dong (University of Sydney, Australia). To promote cell adhesion, cell culture plates were coated with Poly-L-lysine (0.01% in PBS), 2 h prior to cell plating. Cells were grown as monolayers in a humidified incubator at 37 °C with 5% CO_2 atmosphere up to ~80% confluence. Unless stated otherwise, PC-3 cells were cultured in RPMI Medium 1640 + GlutaMAX-1, containing 10% (v/v) foetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml).

Luciferase reporter assay for determining Hedgehog pathway activity. On day 0, PC-3 cells were plated in 24-well plates at a density of 50,000 cells/well. On day 1, when between 70% and 90% confluent, cells were transfected for 24 h with Lipofectamine 2000 (2 μL/well) according to the manufacturer's instructions (without antibiotics). The 8×GBS-luciferase or 8×mutGBS-luciferase reporter plasmids were transfected at 250 ng/well. The phRL-TK *Renilla* internal control plasmid (25 ng/well) was co-transfected for normalisation of transfection efficiency. As a positive control, a plasmid encoding the Gli transcription factor (pcDNA3.1-His-Gli) was transfected (125–500 ng/well) in some conditions. A total of 0.8 μg of plasmid DNA (made up with empty vector, pcDNA3.1) was used per well. All conditions were performed in triplicate. Cells were treated for 24 h with the indicated concentrations of 25-hydoxycholesterol, cyclopamine, or tomatidine. In some experiments, we treated the cells in a low sterol condition (0.5% lipoprotein-deficient serum instead of 10% foetal bovine serum). Luciferase assays were performed using the Dual Luciferase Assay Reporter system according to the manufacturer's instructions in a Veritas luminometer (Turner Designs). Results were expressed as changes in luciferase activity relative to *Renilla* activity, and normalised to the Gli positive control condition performed in each experiment.

Cell viability assay. PC-3 cells were plated at 10,000 cells/well in a 96-well plate in 100 μL of medium. The following day, cells were washed with sterile phosphate-buffered saline and treated with tomatidine or cyclopamine (0–10 μM) for 72 h. The eukaryotic antibiotic, G418 (0.8 mg/mL), was included as a control condition. Each condition was performed in triplicate. Cells were then assayed for cell viability using the WST-1 assay by adding 10 μL of the reagent to the cell suspension (1 in 10 dilution) and incubating for 2 h in a humidified incubator at 37 °C with 5% CO_2 atmosphere. The absorbance of the WST-1 reaction product was then measured at 450 nm on a Molecular Devices Spectra Max 340 plate reader.

Data presentation. Data are presented as means + SEM from triplicate determinations and are representative of at least two separate experiments.

Results

The Hh-inducible 8×GBS-luciferase reporter, containing eight Gli consensus binding sites, was used as a sensitive measure of autonomous activity of the Hh pathway in the common prostate cancer cell line, PC-3. As a negative control, we used a luciferase reporter with mutations that destroy the core Gli DNA-binding sequence (8×mutGBS-luciferase). As a positive control, we co-transfected increasing amounts of a plasmid encoding Gli protein, and found a concentration-dependent increase in Hh-responsive luciferase activity which was absent in the mutant reporter (Fig. 1A). Importantly, there appears to be no basal Hh pathway activity, because in the absence of transfected Gli the value was comparable for the wild-type and mutant reporters.

To exclude the possibility that the PC-3 cells used in this experiment may have undergone phenotypic change(s) due to repeated sub-culturing, we also assayed PC-3 cells which had a lower passage number. The lowest concentration of Gli plasmid from Fig. 1A was employed as a positive control. The basal Hh pathway was at the levels of detection for both the higher and lower passage cells (Fig. 1B and C, respectively), indicating the absence of detectable autocrine signalling in PC-3 cells. The lower passage cells were used in subsequent experiments.

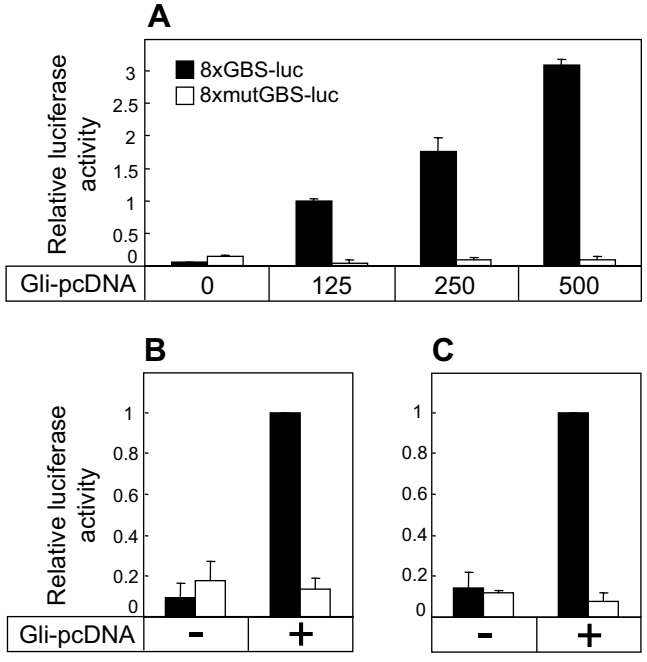

Fig. 1. Neither higher nor lower passage PC-3 cells displayed detectable Hh-inducible Gli transcriptional activity. Cells were transfected with either 8×GBS-luciferase or 8×mutGBS-luciferase, together with increasing amounts of gli-pcDNA (0–500 ng/well). (A,B) Data from higher passage cells. (C) Data from lower passage cells. For (B) and (C), 125 ng gli-pcDNA/well was transfected, and results were averaged from three separate experiments. Luciferase values are presented relative to the 125 ng gli-pcDNA/well condition, which has been set to 1. Further details are given in Materials and methods.

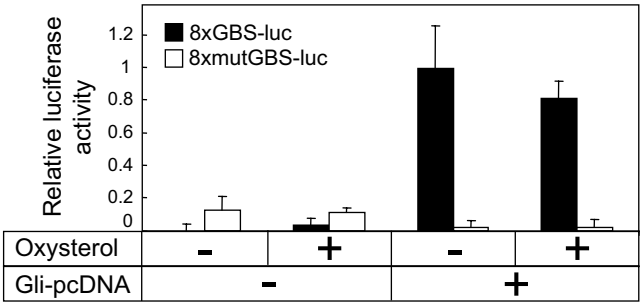

Fig. 2. Oxysterol treatment had no effect on Hh-inducible Gli transcriptional activity in PC-3 cells. Cells were transfected with either 8×GBS-luciferase or 8×mutGBS-luciferase, with or without gli-pcDNA (125 ng/well). After transfection, cells were treated with the oxysterol, 25-hydoxycholesterol (2.5 μM) or vehicle control (ethanol) for 24 h in low sterol medium (containing 0.5% lipoprotein-deficient serum). Luciferase values are presented relative to the 125 ng gli-pcDNA/well condition (without oxysterol), which has been set to 1. Further details are given in Materials and methods.

To further investigate if there is any significant basal activity in PC-3 cells, we employed a manipulation that has been shown to increase Hh pathway activity. Side-chain oxygenated sterols, such as 25-hydroxycholesterol, activate the Hh pathway through the Smoothened protein [15,16]. However, addition of 25-hydroxycholesterol had no effect on Hh pathway activity in the PC-3 cells, as assessed by the 8×GBS-luciferase assay, in that luciferase values were no different between the wild-type and mutant reporters (Fig. 2). This experiment was conducted in low sterol (0.5% lipoprotein-deficient) media in order to maximise the chances of observing an effect of the added sterol. The concentration of 25-hydroxycholesterol used is known to have a profound effect on parameters related to cholesterol homeostasis [17]. We obtained the same negative result in regular (10%) full serum containing media.

The converse approach was to attempt to decrease any Hh pathway activity in the PC-3 cells by treating with the Smoothened inhibitor, cyclopamine. As a control, we used tomatidine, a steroidal alkaloid structurally similar to cyclopamine but which is non-teratogenic and lacks inhibitory effects on Hh signalling. Neither compound had an effect on Hh pathway activity, as again determined by the 8×GBS-luciferase assay (Fig. 3).

Others have found that cyclopamine kills PC-3 cells [6–8], lending support to the contention that these cells possess an active Hh pathway. We treated PC-3 cells with cyclopamine *versus* tomatidine and found no evidence of cytotoxicity after 72 h, using an assay based on reduction of the tetrazolium salt WST-1 by

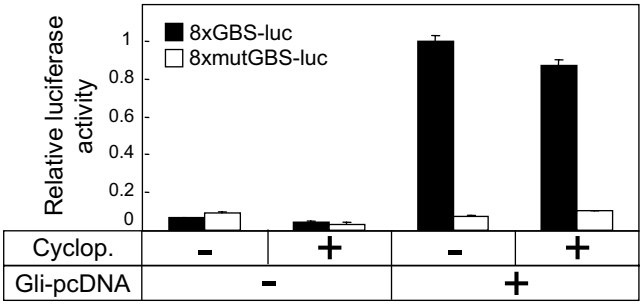

Fig. 3. Cyclopamine treatment had no effect on Hh-inducible Gli transcriptional activity in PC-3 cells. Cells were transfected with either 8×GBS-luciferase or 8×mutGBS-luciferase, with or without gli-pcDNA (125 ng/well). After transfection, cells were treated with cyclopamine (10 μM) or inactive control (10 μM tomatidine) for 24 h. Luciferase values are presented relative to the 125 ng gli-pcDNA/well condition (tomatidine), which has been set to 1. Further details are given in Materials and methods.

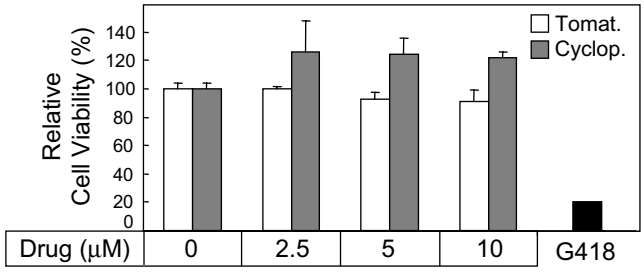

Fig. 4. Cyclopamine treatment had no effect on PC-3 cell viability. Cells were treated with increasing concentrations of cyclopamine or tomatidine for 72 h. 800 μg/mL of G418 was used as a positive control. The WST-1 cell viability assay was performed posttreatment. Values are expressed as a percentage of viable cells where the untreated cells were set to 100%. Further details are given in Materials and methods.

mitochondrial dehydrogenases in viable cells. In contrast, the antibiotic G418 greatly reduced cell viability in the same experiments (Fig. 4).

Discussion

Expression studies in prostate tissue samples and prostate cancer cell lines have presented a strong case for the importance of Hh signalling in prostate cancer [2]. However, the presence of Hh pathway components or Hh gene targets does not necessarily indicate an active Hh pathway. For instance, there are mechanisms that could induce activation of the pathway even in the absence of the Hh ligand, such as activating mutations in Patched or Smoothened [2]. Detection of Gli driven transcription, downstream of Hh signalling, captures the activity along the length of the Hedgehog pathway. Therefore, we argue that the 8×GBS-luciferase reporter assay may be a more accurate reflection of an intact Hh pathway, rather than inferring from components of the pathway being expressed. The 8×GBS-luciferase assay was used by Karhadkar et al. [6] to detect autocrine Hh activity in a number of cell lines, including PC-3 cells. Therefore, the unresponsiveness of this assay for PC-3 cells was unexpected and contradicted the findings of Karhadkar et al. [6]. However, unlike Karhadkar et al. [6], we employed a negative control in which the Gli-binding sites in the luciferase construct were mutated. This proved crucial in determining true basal levels of luciferase activity. Co-transfection with a plasmid encoding Gli confirmed that the assay was working. In addition, manipulations that should either increase (an oxysterol) or decrease (cyclopamine) Hh pathway activity had no effect on 8×GBS-luciferase reporter activity. Overall, we found no evidence of autocrine Hh activity in the commonly used prostate cancer cell line, PC-3, using a sensitive measure of the Hh signalling pathway.

Furthermore, we found that cyclopamine treatment did not affect PC-3 cell viability. Some [6–8,18] but not all [19] studies have found that cyclopamine reduced PC-3 cell viability or proliferation. For example, Karhadkar et al. [6] reported that 1 μM cyclopamine reduced PC-3 viability by >80%. However, Zhang et al. [10] using 10 μM cyclopamine reported a more modest reduction in PC-3 cell viability (~30% after 4 d), but found no effect on Hh-responsive gene expression, indicating that the cyclopamine effect was independent of Hh pathway activity. Our results are consistent with those of Zhang et al. [10], in that we observed no loss in PC-3 cell viability after a shorter incubation with 10 μM cyclopamine (3 d *versus* 4 d). Moreover, Zhang et al. [10] concluded that there is no functional Hh pathway in PC-3 cells, since introduction of Smoothened failed to induce downstream targets. They rationalised Karhadkar et al.'s dramatic finding [6] that xenograft tumours derived from PC-3 cells grew more slowly and even regressed upon

cyclopamine treatment, by suggesting that the cyclopamine may be affecting responses of stromal cells to the xenograft [10].

In conclusion, we found no evidence of autonomous Hh signalling in the widely used prostate cancer cell line, PC-3. These findings are contrary to those from the influential study of Karhadkar et al. [6], using the same assay but incorporating additional controls. Our results support the recent work of Zhang et al. [10], using an alternative approach. Therefore, we caution against using PC-3 cells to investigate the Hh pathway in a prostate cancer setting.

Acknowledgments

We thank the following researchers for generously sharing their tools: Dr. Hiroshi Sasaki (Center for Developmental Biology, RIKEN Kobe, Japan) for his Gli plasmids; Professor Pam Russell (Oncology Research Unit, The Prince of Wales Hospital, Sydney, Australia) for the higher passage PC-3 cells; Dr. Qihan Dong (University of Sydney, Australia) for the lower passage PC-3 cells. We thank members of the Brown lab for critically reading this manuscript. AJB's research is supported by a grant from the Prostate Cancer Foundation of Australia (PR36).

References

[1] D.H. Barnett, H.Y. Huang, X.R. Wu, R. Laciak, E. Shapiro, W. Bushman, The human prostate expresses sonic hedgehog during fetal development, J. Urol. 168 (2002) 2206–2210.
[2] S. Datta, M.W. Datta, Sonic Hedgehog signaling in advanced prostate cancer, Cell. Mol. Life Sci. 63 (2006) 435–448.
[3] A. Shaw, W. Bushman, Hedgehog signaling in the prostate, J. Urol. 177 (2007) 832–838.
[4] T. Osterlund, P. Kogerman, Hedgehog signalling: how to get from Smo to Ci and Gli, Trends Cell Biol. 16 (2006) 176–180.
[5] A.J. Brown, Cholesterol, statins and cancer, Clin. Exp. Pharm. Physiol. 34 (2007) 135–141.
[6] S.S. Karhadkar, G.S. Bova, N. Abdallah, S. Dhara, D. Gardner, A. Maitra, J.T. Isaacs, D.M. Berman, P.A. Beachy, Hedgehog signalling in prostate regeneration, neoplasia and metastasis, Nature 431 (2004) 707–712.
[7] P. Sanchez, A.M. Hernandez, B. Stecca, A.J. Kahler, A.M. DeGueme, A. Barrett, M. Beyna, M.W. Datta, S. Datta, A. Ruiz i Altaba, Inhibition of prostate cancer proliferation by interference with SONIC HEDGEHOG-GLI1 signaling, Proc. Natl. Acad. Sci. USA 101 (2004) 12561–12566.
[8] T. Sheng, C. Li, X. Zhang, S. Chi, N. He, K. Chen, F. McCormick, Z. Gatalica, J. Xie, Activation of the hedgehog pathway in advanced prostate cancer, Mol. Cancer 3 (2004) 29.
[9] G. Shaw, A.M. Price, E. Ktori, I. Bisson, P.E. Purkis, S. McFaul, R.T. Oliver, D.M. Prowse, Hedgehog signalling in androgen independent prostate cancer, Eur. Urol. (2008).
[10] 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.
[11] J. Gipp, G. Gu, C. Crylen, S. Kasper, W. Bushman, Hedgehog pathway activity in the LADY prostate tumor model, Mol. Cancer 6 (2007) 19.
[12] J.L. Goldstein, S.K. Basu, M.S. Brown, Receptor-mediated endocytosis of low-density lipoprotein in cultured cells, Methods Enzymol. 98 (1983) 241–260.
[13] H. Sasaki, C. Hui, M. Nakafuku, H. Kondoh, A binding site for Gli proteins is essential for HNF-3beta floor plate enhancer activity in transgenics and can respond to Shh in vitro, Development 124 (1997) 1313–1322.
[14] M.E. Kaighn, K.S. Narayan, Y. Ohnuki, J.F. Lechner, L.W. Jones, Establishment and characterization of a human prostatic carcinoma cell line (PC-3), Invest. Urol. 17 (1979) 16–23.
[15] J.R. Dwyer, N. Sever, M. Carlson, S.F. Nelson, P.A. Beachy, F. Parhami, Oxysterols are novel activators of the hedgehog signaling pathway in pluripotent mesenchymal cells, J. Biol. Chem. 282 (2007) 8959–8968.
[16] R.B. Corcoran, M.P. Scott, Oxysterols stimulate Sonic hedgehog signal transduction and proliferation of medulloblastoma cells, Proc. Natl. Acad. Sci. USA 103 (2006) 8408–8413.
[17] X. Du, Y.H. Pham, A.J. Brown, Effects of 25-hydroxycholesterol on cholesterol esterification and SREBP processing are dissociable: implications for cholesterol movement to the regulatory pool in the endoplasmic reticulum, J. Biol. Chem. 279 (2004) 47010–47016.
[18] R.J. Levitt, Y. Zhao, M.J. Blouin, M. Pollak, 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, Cancer Lett. 255 (2007) 300–306.
[19] J. Sims-Mourtada, J.G. Izzo, J. Ajani, K.S. Chao, Sonic Hedgehog promotes multiple drug resistance by regulation of drug transport, Oncogene 26 (2007) 5674–5679.