Tumor and Stem Cell Biology

Cancer Research

Common Botanical Compounds Inhibit the Hedgehog Signaling Pathway in Prostate Cancer

Anna Ślusarz[1,6], Nader S. Shenouda[1,6], Mary S. Sakla[2,6], Sara K. Drenkhahn[1,6], Acharan S. Narula[7], Ruth S. MacDonald[6,8], Cynthia L. Besch-Williford[3,6], and Dennis B. Lubahn[1,4,5,6]

Abstract

Many botanical compounds have been proposed to prevent cancer. We investigated the cancer treatment and prevention abilities of apigenin, baicalein, curcumin, epigallocatechin 3-gallate (EGCG), genistein, quercetin, and resveratrol both *in vivo* in transgenic adenocarcinoma of the mouse prostate (TRAMP) mice as well as *in vitro* in prostate cancer cell lines. In our experiments, these seven compounds act similarly to the Hedgehog antagonist cyclopamine, a teratogenic plant alkaloid, which had been previously shown to "cure" prostate cancer in a mouse xenograft model. With IC_{50} values ranging from <1 to 25 μmol/L, these compounds can inhibit *Gli1* mRNA concentration by up to 95% and downregulate Gli reporter activity by 80%. We show that four compounds, genistein, curcumin, EGCG, and resveratrol, inhibit Hedgehog signaling as monitored by real-time reverse transcription-PCR analysis of *Gli1* mRNA concentration or by Gli reporter activity. Three compounds, apigenin, baicalein, and quercetin, decreased *Gli1* mRNA concentration but not Gli reporter activity. Our results show that these compounds are also able to reduce or delay prostate cancer *in vivo* in TRAMP mice. All seven compounds, when fed in combination as pure compounds or as crude plant extracts, inhibit well-differentiated carcinoma of the prostate by 58% and 81%, respectively. *In vitro*, we show that all seven compounds also inhibit growth in human and mouse prostate cancer cell lines. Mechanistically, we propose the Hedgehog signaling pathway to be a direct or indirect target of these compounds. These botanicals at pharmacologic concentrations are potentially safer and less expensive alternatives to cyclopamine and its pharmaceutical analogues for cancer therapy. *Cancer Res; 70(8); 3382–90. ©2010 AACR.*

Introduction

Prostate cancer remains the second most commonly diagnosed cancer in the United States. According to the Prostate Cancer Foundation, one of every three men diagnosed with cancer will be diagnosed with prostate cancer. It is also the second leading cause of cancer deaths of men in the United States. Because prostate cancer typically develops later in life, identifying botanical compounds that delay the progression of this disease will have a positive effect on quality of life and reduce healthcare costs of the aging population.

Authors' Affiliations: [1]Department of Biochemistry, [2]Genetics Area Program, [3]Department of Veterinary Pathobiology, [4]Department of Animal Sciences, [5]Department of Child Health, and [6]MU Center for Phytonutrient and Phytochemical Studies, University of Missouri, Columbia, Missouri; [7]Narula Research, Chapel Hill, North Carolina; and [8]Department of Food Science and Human Nutrition, Iowa State University, Ames, Iowa

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Corresponding Author: Dennis B. Lubahn, University of Missouri, 110 ASRC, 920 East Campus Drive, Columbia, MO 65211. Phone: 573-884-6781; Fax: 573-882-6827; E-mail: lubahnd@missouri.edu.

doi: 10.1158/0008-5472.CAN-09-3012

It is well known that diet and other environmental factors can greatly reduce the risk of cancer incidence. In particular, dietary phytoestrogens and antioxidants have been implicated in protecting against cancer (1). We have selected a group of seven botanical compounds that have been reported to have prostate cancer–protective activities (2) and have been widely used in traditional medicine and in dietary supplements that are currently available in the United States (3). Those compounds include apigenin from *Matricaria recutita* (chamomile), baicalein from *Scutellaria baicalensis* Georgi (Chinese skullcap), curcumin from *Curcuma longa* (turmeric), epigallocatechin 3-gallate (EGCG) from *Camellia sinensis* Kuntze (green tea), genistein from *Glycine max* (soy), quercetin from *Ginkgo biloba*, and resveratrol from *Vitis vinifera* (grape).

We have previously reported the cancer-preventive effect of each of these seven compounds in PC3 and LNCaP human prostate cancer cell lines (1). Here, we show that all seven can also individually inhibit growth of the mouse prostate cancer cell line TRAMP-C2 (4).

Pursuant to these results, we wanted to determine whether these seven botanical compounds would affect the Hedgehog signaling pathway, which, through its inhibitor cyclopamine, has been recently found to be important in prostate cancer and its treatment (5–8).

The Hedgehog signal transduction pathway is crucial to the growth, survival, and organization of many cells, tissues, and organs. Dysregulation of the Hedgehog signal transduction pathway has been implicated in several cancers, including human prostate cancer (6–9). The pathway is activated by one of three types of Hedgehog protein: Sonic, Desert, or Indian. These are secreted proteins that bind to their membrane receptors, Patched1 or Patched2, which in turn relieve the inhibition of another transmembrane protein, Smoothened. Smoothened mediates its actions via three transcription factors in the Gli family, specifically Gli1, Gli2, and Gli3 proteins. Several types of cancer, including prostate cancer, show greatly increased Hedgehog pathway activation. Therefore, inhibition of Gli function might be a promising prevention and therapeutic target in certain tumors.

We found that four of the seven botanical compounds can inhibit the Hedgehog signaling pathway *in vitro* both in the prostate cancer TRAMP-C2 cells as well as in an established Hedgehog pathway assay in Sonic Hedgehog (Shh) Light II cells. We propose that the prostate cancer–preventative effects of these dietary botanicals may result from inhibition of the Hedgehog pathway and that they potentially represent an inexpensive, safe, and effective alternative to cyclopamine in cancer prevention and treatment.

Materials and Methods

TRAMP mouse studies. Male TRAMP mice (10) on a C57BL6/J background were raised in-house as described previously (11). The mice were fed the specific diets from 5 wk until 5 mo of age (18–22 mice per treatment group). Prostates were collected, formalin fixed, and paraffin embedded. Tissue sections were stained with H&E and examined by light microscopy for assessment of cancer stages (12).

For diet formulations, see Supplementary Table S1.

Cell culture. All cell lines were obtained from the American Type Culture Collection. Mouse prostate cancer TRAMP-C2 and human prostate cancer PC3 cell lines were maintained in RPMI 1640 supplemented to contain 10% fetal bovine serum (FBS; U.S. Bio-Technologies), 4.5 g/mL glucose, 4 mmol/L L-glutamine, 100 μmol/L nonessential amino acids, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, and 1% penicillin/streptomycin (all from Invitrogen).

Shh Light II cells (JHU-68) were maintained in DMEM with 4 mmol/L L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose supplemented with 0.4 mg/mL G-418, 0.15 mg/mL zeocin (Invitrogen), and 10% calf serum. This mouse embryonal NIH 3T3 cell line contains a stably transfected luciferase reporter with eight copies of the consensus Gli binding site derived from the mouse hepatocyte nuclear factor-3β (JHU-73 pGL3B/8XgliBS-lc-luc 5′-GAACACCCA-3′; ref. 13).

The purified compounds used in tissue culture experiments were obtained from the following suppliers: apigenin (LC Laboratories), baicalein (Indofine Chemical Co.), curcumin (Sigma), cyclopamine (Toronto Research Chemicals and LC Laboratories), EGCG (Sigma), genistein (Sigma), quercetin (Sigma), resveratrol (Sigma). For structures, see Supplementary Fig. S1.

Mouse recombinant Shh was obtained from R&D Systems. All compounds were dissolved in DMSO or ethanol; Shh was dissolved in PBS with 0.1% bovine serum albumin. In each experiment, the controls and all treatments contained all vehicles used. All treatments were conducted in phenol red–free medium with charcoal-stripped serum.

Protein assay. All compound treatments for the growth assessment consisted of a 72-h time course in phenol red–free RPMI 1640 supplemented with 10% charcoal-stripped FBS. Cells were seeded in 12-well plates and adjusted to phenol red–free medium for 24 h. Cells were lysed with 1 N NaOH and left overnight. Protein assays to measure overall cell protein concentration were performed using Bio-Rad DC kit, with absorbance measured at 750 nm. Compounds were used at concentrations ranging from 1 to 100 μmol/L in half-log increments. Each experiment was performed at least thrice in duplicate. Total cellular protein correlates well with thymidine uptake in prostate cancer cells and is a reliable assay to measure cell growth (1).

RNA isolation. Total RNA was isolated from the TRAMP-C2 and PC3 cell lines using the RNeasy kit (Qiagen). RNA concentration was determined using the ND 1000 Spectrophotometer v3.1 (NanoDrop Technologies).

Real-time reverse transcription-PCR. Relative expression of mRNA was measured by one-step real-time reverse transcription-PCR (RT-PCR) with Taqman EZ RT-PCR kit (Applied Biosystems) on the Applied Biosystems 7500 Real-Time PCR System. For primer sequences and reaction conditions, see Supplementary Table S2. Each reaction was performed in triplicate on at least three individually isolated RNA samples per treatment. Data were analyzed using the $\Delta\Delta C_T$ method (14).

Reporter assay. Gli activity in the Shh Light II cell line was assayed after 48 h of treatment with selected compounds in phenol red–free DMEM supplemented with 0.5% charcoal-stripped serum using the Dual-Luciferase Reporter Assay System (Promega). Each experiment was performed at least thrice in duplicate.

Western blot analysis. Cells were lysed using passive lysis buffer (Epitomics), scraped on ice, sonicated, or passed through a 26-gauge needle and spun for 30 min 16,000 × *g* at 0°C. Cell lysates were collected and stored at −80°C. Mouse prostates were disrupted in liquid N_2 or using a tissue homogenizer in TEG buffer [10 mmol/L Tris, 1.5 mmol/L EDTA, 10% glycerol (pH 7.4)]. Tissues were spun for 10 min at 300 × *g* at 4°C, and lysates were spun again for 30 min at 16,000 × *g* at 0°C. Tissue lysates were collected and stored at −80°C.

Rabbit polyclonal antibody against mouse Gli1 (Abcam and Biodesign) and against β-actin (C4; Santa Cruz Biotechnology) was used according to the manufacturer's recommendations. Western blot bands were scanned to Adobe Photoshop.

Statistical analysis. GraphPad Prism 4 (GraphPad Software) was used to calculate *P* values.

In vivo studies were analyzed with the χ^2 test, comparing each treatment versus control diet for each cancer

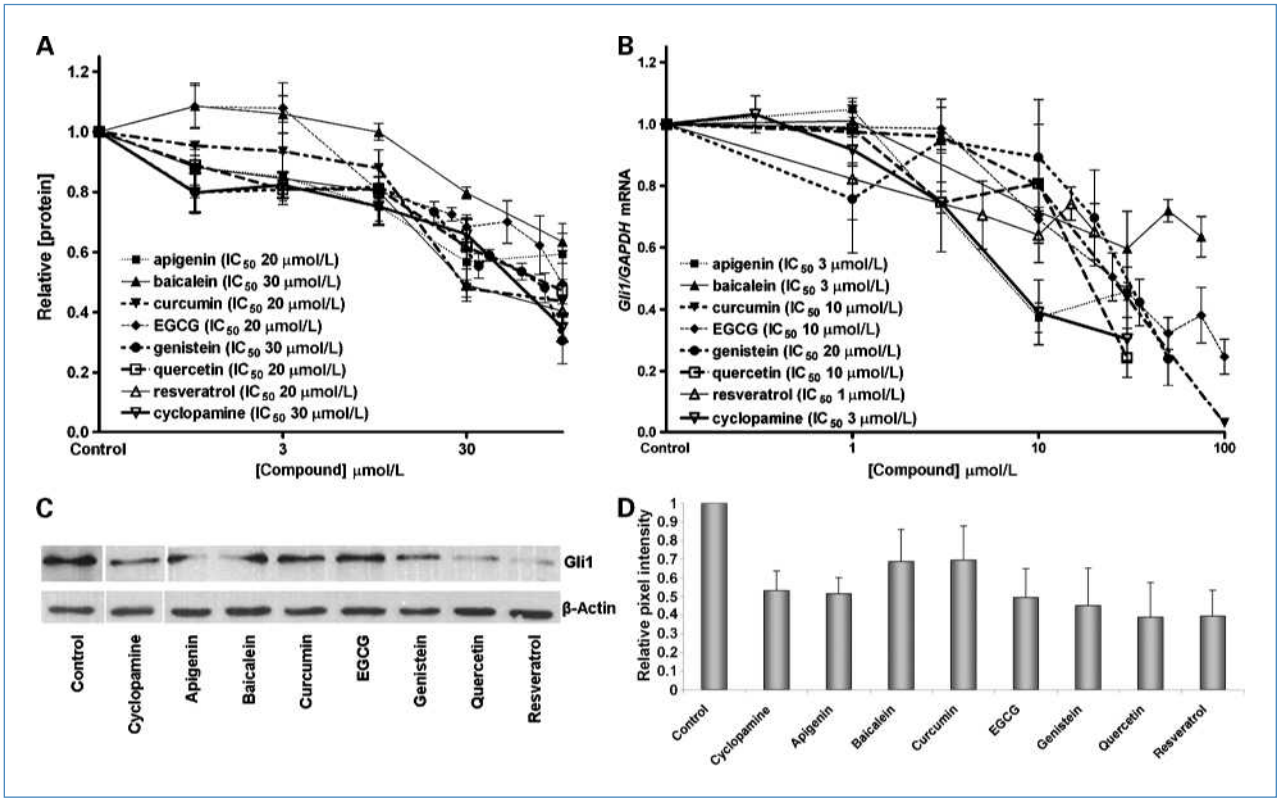

Figure 1. Botanical compounds inhibit cell growth and Gli1 expression in TRAMP-C2 cells. A, mouse prostate cancer cell growth was determined based on total protein concentration after a 72-h treatment with each compound relative to control treatment. Each experiment was performed at least thrice in duplicate. IC_{50} values represent the concentration of a compound at which we observed half-maximal inhibition for this compound with a baseline correction. For individual compounds, see Supplementary Fig. S2. B, *Gli1/GAPDH* mRNA concentrations indicating Hedgehog pathway activity after 72 or 24 h (curcumin and quercetin) of treatment with various botanicals were determined by real-time RT-PCR. For summary, see Supplementary Table S3. C, representative Western blots using Gli1 antibody from Biodesign. Each compound was used at 10 μmol/L for 24 h. For additional Western blot of apigenin, baicalein, and EGCG, see Supplementary Fig. S8. D, quantification of Western blot bands by total pixel Gli1/actin. Columns, average of independent experiments; bars, SD.

stage. *In vitro* results were analyzed using the *t* test. A *P* value of <0.05 was considered statistically significant.

Results

Botanical compounds inhibit TRAMP-C2 cell growth. All of the seven botanical compounds tested were able to inhibit cell growth in the mouse prostate cancer cell line TRAMP-C2, with IC_{50} values between 20 and 30 μmol/L (Fig. 1A; Supplementary Fig. S2). Treatments of the compounds ranging from 1 to 100 μmol/L resulted in maximal inhibitions varying from 25% and 70%, with genistein showing the strongest effect followed by cyclopamine > curcumin = resveratrol > quercetin > EGCG > baicalein > apigenin. The inhibition of total cellular protein by genistein is significant starting at concentrations as low as 1 μmol/L.

Botanical compounds decrease basal and Shh-stimulated* Gli1 *mRNA in TRAMP-C2 cells. The botanical compounds inhibited Hedgehog signaling, measured by real-time RT-PCR analysis of basal *Gli1* mRNA concentrations, in TRAMP-C2 cells after 24 and 72 hours of treatments (Figs. 1B and 2A and B). With an IC_{50} of <1 μmol/L, resveratrol was the most potent, followed by apigenin, baicalein, and cyclopamine. Curcumin was the most effective, reaching a maximal inhibition of 95%, followed by cyclopamine with 85%. Baicalein and resveratrol reduced *Gli1* concentrations the least, only reaching 35% maximal inhibition at 30 and 10 μmol/L, respectively. In each case, the IC_{50} values for *Gli1* mRNA reduction were lower than the IC_{50} values for growth inhibition, indicating that multiple pathways may need to be altered to inhibit cell growth.

It has been reported that a functional primary cilia is required for the Hedgehog pathway to be active (15). As cells in culture do not develop a primary cilium until they become confluent (16), we performed a time course experiment with cyclopamine and genistein, starting with confluent cells. Because all seven compounds inhibited TRAMP-C2 cell growth after a 72-hour treatment and decreased Gli1 protein concentrations in TRAMP-C2 cells after a 24-hour treatment (Figs. 1C and D and 2C), we analyzed the Hedgehog pathway to determine if the inhibition was an apparent secondary effect due to reduced cell confluency. Beginning near 100% confluency and investigating the cells after 2-, 4-, 8-, 24-, and 72-hour time points, we were able to observe a rapid

inhibition of Hedgehog activity, thus excluding the possibility of an indirect growth retardation effect of the compounds. Cyclopamine (30 μmol/L) was able to cause a significant decrease in *Gli1* mRNA concentration after only 2 hours of treatment and 50 μmol/L genistein decreased *Gli1* mRNA significantly after 4 hours (Fig. 2D). These results confirm that the relatively fast-acting Hedgehog-inhibitory ability of genistein is independent of growth and a subsequent change in cilia or cell confluency.

We next tested whether *Gli1* mRNA expression in the TRAMP-C2 cell line could be increased with the pathway agonist Shh. Treatment of TRAMP-C2 cells with 0.5 μg/mL NH_2-terminal Shh peptide caused a 25-fold elevation in *Gli1* mRNA. Both 3 μmol/L cyclopamine and 5 μmol/L genistein were able to reproducibly decrease this stimulated *Gli1* mRNA (Fig. 3A and B).

Botanical compounds decrease Shh-stimulated Gli reporter activity in Shh Light II cells. To independently confirm the Hedgehog pathway inhibition of the seven botanical compounds, we tested them in the Hedgehog-responsive fibroblast cell line Shh Light II (13, 17). Four of the seven compounds were able to decrease Shh peptide-stimulated Gli reporter activity. The cyclopamine positive control had the strongest effect at 30 μmol/L, followed by curcumin, EGCG, genistein, and resveratrol. Apigenin, baicalein, and quercetin were not able to inhibit the pathway in this system (Fig. 4).

To test the general significance of our observations, we selected genistein, based on cost, availability, and purity, to analyze further its functionality in a human prostate cancer cell line, PC3. Genistein can significantly inhibit PC3 growth starting at 10 μmol/L (Supplementary Fig. S3A), with an IC_{50} of 40 μmol/L. At 10 μmol/L, genistein is also able to significantly inhibit *Gli1* mRNA concentrations in the PC3 cells by >50% (Supplementary Fig. S3B).

Botanical compounds prevent tumorigenesis in TRAMP mice. Due to economical reasons, we were not able to test each compound individually *in vivo*. For these initial studies, the compounds were grouped based on their structure. The nonflavones/nonisoflavones curcumin and resveratrol

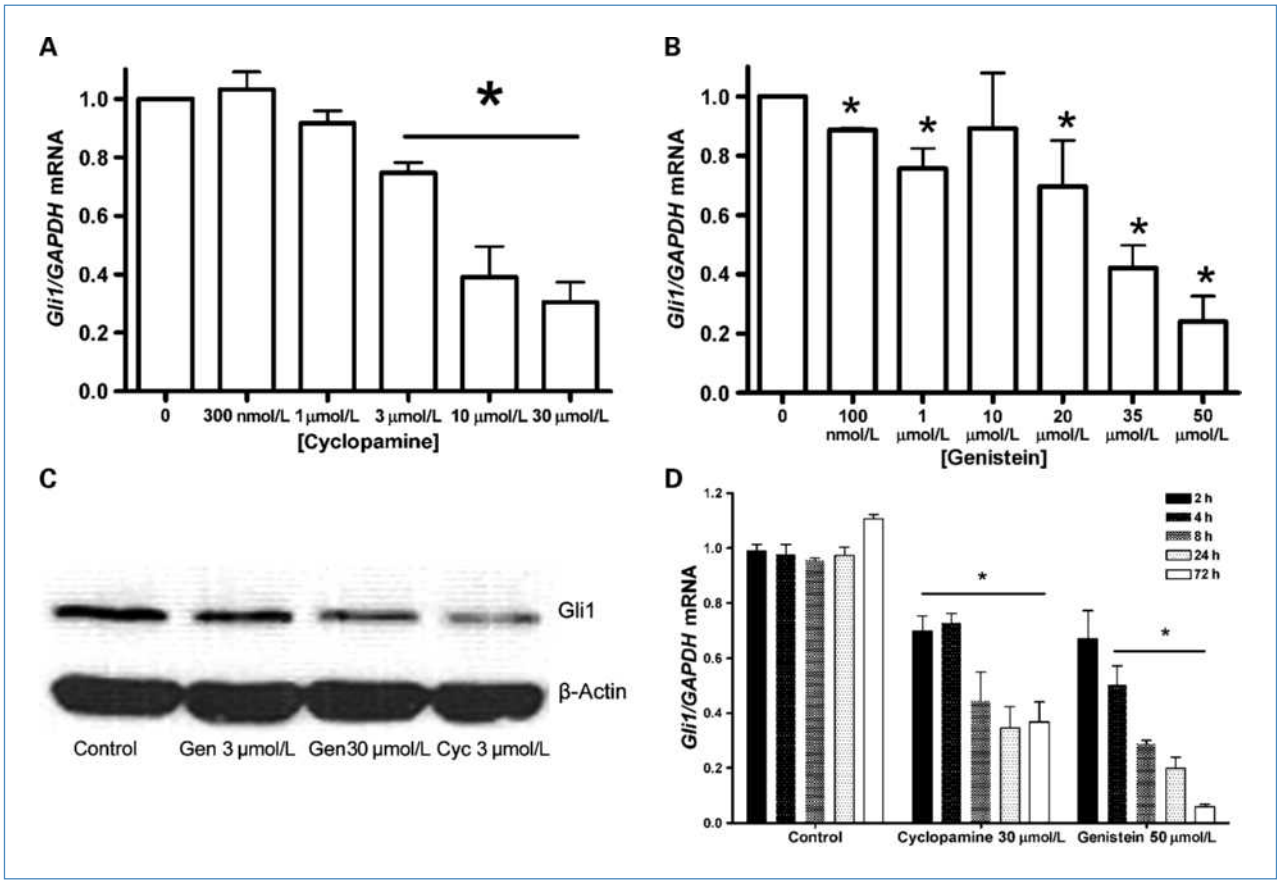

Figure 2. Cyclopamine and genistein inhibit Hedgehog pathway activity in TRAMP-C2 cells. Relative *Gli1* mRNA concentrations after 72 h of cyclopamine (A) and genistein (B) treatment as determined by real-time RT-PCR. C, Gli1 protein decreases after 24 h of treatment with 3 μmol/L cyclopamine (Cyc) and 3 and 30 μmol/L genistein (Gen). D, genistein and cyclopamine can inhibit *Gli1* mRNA in TRAMP-C2 in 4 and 2 h, respectively. Subconfluent TRAMP-C2 cells were treated with 50 μmol/L genistein or 30 μmol/L cyclopamine, and RNA was isolated after 2, 4, 8, 24, and 72 h. *Gli1* mRNA was measured by real-time RT-PCR relative to *GAPDH* mRNA. Each experiment was performed at least thrice in duplicate. *t* test was performed to determine *P* value. *, $P < 0.05$. Bars, SD. For a comparison between the positive control Hedgehog inhibitor cyclopamine and the negative control structurally related tomatidine, see Supplementary Fig. S7.

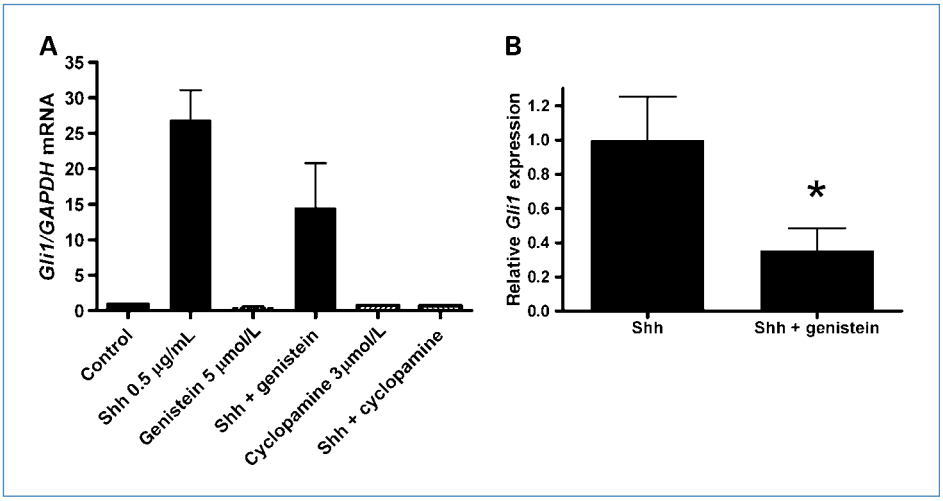

Figure 3. Shh can stimulate *Gli1* mRNA concentrations in TRAMP-C2 cells, and genistein can inhibit the stimulated *Gli1* expression. A, TRAMP-C2 cells were treated with mouse recombinant 0.5 μg/mL Shh for 24 h. Cotreatment with 5 μmol/L genistein resulted in significant reduction of *Gli1* mRNA concentrations. Shh stimulation varied significantly between experiments. B, genistein inhibition of Shh-stimulated *Gli1* expression normalized to 0.5 μg/mL Shh–stimulated state within individual experiments because the stimulation by Shh varied between 3- and 8-fold between experiments. Each experiment was performed at least thrice in duplicate. *t* test was performed to determine *P* value. *, $P < 0.05$. Bars, SD.

plus EGCG were used together in Pure 3 Diet. The flavones/isoflavones apigenin, baicalein, genistein, and quercetin were used together in Pure 4 Diet (for structures, see Supplementary Fig. S1). To examine combinatorial effects, all seven pure compounds were used in the Pure 7 Diet. Crude plant materials or extracts from which the pure compounds were derived were combined in the Crude 7 Diet to further explore natural exposure to these compounds. Saw palmetto was chosen in lieu of *S. baicalensis* because of initial difficulties in obtaining the latter. We tested the combinations of compounds used in the diets in TRAMP-C2 cells, with the concentration of each compound slightly below the concentration that caused significant inhibition of *Gli1* mRNA. When combined at these concentrations, the tested mixes resulted in significant reduction of *Gli1* mRNA (Supplementary Fig. S4).

TRAMP mice fed the experimental diets grew similarly and consumed similar amounts of each diet throughout the study, with no group weight mean varied >7% from overall weight mean (Supplementary Fig. S5). At 5 months of age, prostates were staged according to severity of lesions (12). As observed with earlier studies, no normal prostates were seen at this age, and a wide range of cancer stages was present. The relative incidence hyperplasia, prostatic intraepithelial neoplasia (PIN), well-differentiated carcinoma (WDC), moderately differentiated carcinoma (MDC), and poorly differentiated carcinoma (PDC) were quantified by histologic examination by a trained veterinary pathologist who was blinded to the treatments. All experimental diets significantly decreased overall cancer incidence, defined as WDC, MDC, and PDC, when compared with control diet (Table 1). Within the specific cancer stages, all diets decreased WDC incidence in the TRAMP mice, with Pure 4 Diet (apigenin, baicalein, genistein, and quercetin) and Crude 7 Diet having the strongest effect (Table 2).

Discussion

Four articles were published in the fall of 2004 that profoundly altered the outlook for prostate cancer treatment (5–8). The authors independently reported that advanced

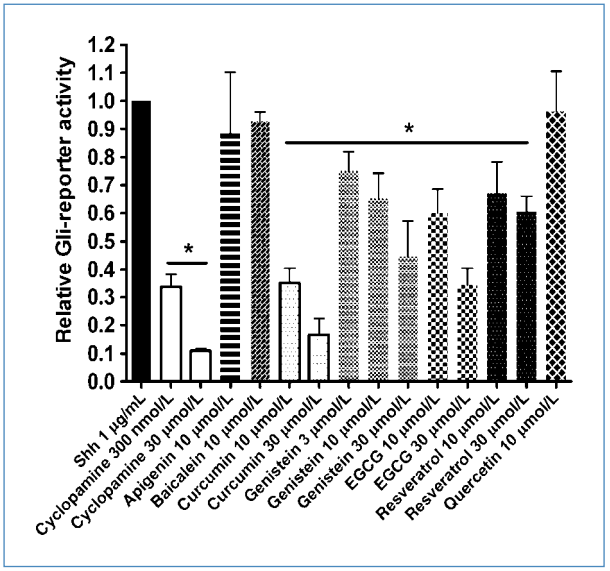

Figure 4. Botanical compounds inhibit Shh-stimulated Gli-responsive promoter in the Shh Light II cell line. Shh Light II cells were treated with various compounds in the presence of 1 μg/mL Shh for 24 h. Each experiment was performed in triplicate at least thrice. *t* test was performed to determine *P* value. *, $P < 0.05$. Bars, SD.

Table 1. Incidence of prostate tumorigenesis in TRAMP mice fed various botanical compounds

Diet	*n*	Phenotype, *n* (%)	
		Noncancer	Cancer
Casein	22	3 (13.6%)	19 (86.4%)
Pure 3	20	10 (50%)	10 (50%)*
Pure 4	18	12 (66.7%)	6 (33.3%)†
Pure 7	19	11 (57.8%)	8 (42.2%)‡

NOTE: Male TRAMP mice were started on diets at weaning and sacrificed at 5 mo. Pure 3 = curcumin + resveratrol + EGCG; Pure 4 = apigenin + baicalein + genistein + quercetin; Pure 7 = Pure 3 + Pure 4. Noncancer was defined as normal, hyperplasia, and PIN. Cancer was defined as WDC, MDC, and PDC. χ^2 Test was performed to compare cancer versus noncancer incidence for each treatment diet versus casein control diet.

*$P < 0.05$.

†$P < 0.001$.

‡$P < 0.01$.

human prostate cancer specimens and metastases showed elevated Hedgehog pathway activity. These studies found that cyclopamine, an alkaloid isolated from *Californium veratrum*, was able to inhibit human prostate cancer cell proliferation *in vitro* and was able to cure prostate cancer mouse xenograft models, making cyclopamine a potentially promising treatment for prostate cancer (5–7, 18). The high cost of cyclopamine, however, makes it an unrealistic drug for wide-scale use (19). Currently, several companies are working to develop small-molecule Hedgehog pathway antagonists, some of which are now entering phase 2 clinical trials.

We were interested in testing cyclopamine in our TRAMP mice to see whether it was able to cure SV40 T/t antigen–induced prostate tumors *in vivo*. Unfortunately, the price and availability of cyclopamine at the time made it impossible to conduct extensive animal studies. With the knowledge that our previous investigations revealed that several botanical compounds inhibit prostate cancer cell proliferation *in vitro* (1) and the fact that there are many known possible mechanisms of action for botanical compounds, we decided to test the selected seven compounds for their ability to inhibit Hedgehog signaling. These botanicals at pharmacologic concentrations offer a potentially safer and less expensive alternative to cyclopamine and its pharmaceutical analogues for cancer therapy.

When used in the diet, these compounds delayed prostate cancer incidence *in vivo* in TRAMP mice (Tables 1 and 2). All of the diets led to a significant reduction in overall cancer incidence when compared with the casein control diet. Additionally, there was a significant reduction at the WDC stage with the Pure 4 and Crude 7 Diets (Table 2). After obtaining these results with our combination diets, we next plan to test some of the compounds individually. Genistein (20, 21), EGCG (22–26), resveratrol (27), and apigenin (28) have all been previously tested, and only high-dose genistein (250–500 mg/kg; ref. 21) has been reported to affect PDC. Thus, there is a need to further test curcumin for its effect in cancer prevention when used in the diet, as well for treatment of already established tumors. The effects of cyclopamine and genistein on Hedgehog signaling in TRAMP mice also need to be examined.

We were very interested in testing the effects of the seven compounds on Hedgehog signaling *in vitro* and *in vivo*. For our *in vitro* studies, we used the TRAMP-C2 cell line, derived from a primary prostate tumor of a 32-week-old TRAMP mouse (4), and the Shh Light II cell line. Our results show that four of the seven compounds, genistein, curcumin, EGCG, and resveratrol, inhibited the Hedgehog signaling pathway in both cell lines. It was interesting to see the discrepancy in Hedgehog pathway inhibition between *Gli1* mRNA inhibition in TRAMP-C2 cells and Gli reporter activity in Shh Light II cells. Three of seven compounds, apigenin, baicalein, and quercetin, inhibit *Gli1* mRNA strongly at low concentrations, but we could not observe any inhibition with concentrations as high as 30 μmol/L in the Shh Light II cells.

Potential reasons these three compounds are not inhibiting the Hedgehog signaling pathway in both assays could be due to their direct versus indirect effects on the pathway, with the possibility of cell-specific characteristics from cross-talk of additional unknown pathways with the Hedgehog signaling pathway. There are many significant steps between the Hedgehog signal at the cell membrane and the Gli-regulated transcription response in the nucleus, with a variety of potential signaling interactions not yet fully explored. For example, in melanomas, interactions between Gli1 and the Ras–mitogen-activated protein/extracellular signal-regulated kinase kinase/AKT pathways have been observed (29). Finally, a recent report presents data that in some cell lines Hedgehog pathway activation at the level of Gli1 and Gli2 can be inhibited by transforming growth factor-β inhibitors but not by cyclopamine (30). In addition, all of the targets of botanical compounds have not been completely determined. Genistein, the most extensively studied, regulates a large number of molecular and enzymatic activities, which may interact with Hedgehog signaling (for references, see Supplementary Materials and Methods). The changes in enzyme activities could differentially alter an endogenous inhibitor that is absent in one of the cells, or the different media used to culture the two cell lines could impinge on a cross-talking pathway. Potential candidates for such inhibitors or modulators are cholesterol derivatives and precursors whose concentrations vary widely between cell types and culture media. We are using charcoal-stripped media for all our treatments, with the charcoal removing most cholesterol-related compounds, such as the Hedgehog-activating oxysterols (31, 32), or sex steroids, such as androgens, which also have been shown to inhibit Hedgehog signaling in prostate cancer (33, 34).

Another possibility for the interlab differences found in Hedgehog signaling in prostate cancer cells may be related to the fact that all of our *in vitro* cell culture experiments

have been conducted with cells of epithelial origin. By the current dogma, Hedgehog signaling consists of communication between different cell types. The ligand (Hedgehog) is produced by epithelial cells at mesenchymal interfaces and signals to the adjacent mesenchyme through its receptor Patched (35–37). Many cancer cells undergo an epithelial-mesenchymal transition with cancer progression, which could be what we are seeing in the TRAMP-C2 cells. Tumors from TRAMP mice and TRAMP-C2 cells clearly show Hedgehog signaling, which makes them appropriate for studying prostate cancer responses to Hedgehog antagonists. It had been proposed that tumor cells of epithelial origin would not have a functional autocrine Hedgehog signaling (38, 39); however, our TRAMP-C2 cells show Hedgehog signaling that is both inducible and inhibitable (Fig. 3). Whereas Zhang and colleagues (40) showed a lack of demonstrable autocrine Hedgehog signaling in human prostate cancer cell lines, we have observed cyclopamine inhibiting PC3 and LNCaP cell growth (data not shown), as well as decreased *Gli1* mRNA in PC3 cells by genistein (Supplementary Fig. S3B). These interlab differences could be due to higher starting confluencies (41) or different components in the stripped versus nonstripped cell culture media.

In vivo differences in expression of mouse prostate Hedgehog components have also been observed. In the transgenic LADY model, an alternative prostate cancer model to TRAMP, Gipp and colleagues (42) reported a lack of increased Hedgehog signaling markers during tumor development. The LADY mice have been created using the androgen-regulated probasin driving the large T antigen on a CD-1 background. Although the tumors are very fast growing, they rarely produce metastases (43). This lack of metastatic potential might be explained by the low Hedgehog pathway activity in this model and stress the importance of strain background for studying the pathway. Interestingly, C57BL6 mice carry a Patched polymorphism when compared with FVB mice. This polymorphism makes them highly resistant to development of skin squamous carcinomas (44), and presumably, genetic background strain differences from CD-1 compared with FVB/C57BL6 might also explain the variations in Hedgehog signaling we observe *in vivo* between labs.

When we analyzed tumors from TRAMP mice for *Gli1* mRNA and Gli1 protein by Western blot, we observed a high variance in Gli1 expression in the hyperplasia and PDC stages among and within treatments (Supplementary Fig. S6). This could be due to the heterogeneous nature of our sample materials, as PDC tumors tend to be nonhomogeneous and may contain large necrotic areas. We are planning to perform immunohistochemical analysis on the tissues as soon as a lot-to-lot consistent Gli1 antibody becomes available in larger quantities.

The doses of the botanical compounds that we have shown to be effective *in vitro* are at apparent pharmacologic levels. Dietary genistein concentrations, on the other hand, may be sufficient to raise the concentration of genistein within the prostate to achieve Hedgehog pathway inhibition. Reports on free genistein levels (aglycone) in serum and prostate of rats fed genistein diets vary. Dalu and colleagues (45) reported bioavailable genistein to be comparable between serum and dorsolateral prostate in Lobund-Wistar rats fed 250 mg/kg and 1 g/kg genistein diets at ~18 and 150 nmol/L, respectively. Chang and colleagues (46), however, reported higher concentrations of aglycone genistein in several tissues including the prostate compared with serum. In their hands, Sprague-Dawley rats fed 100 and 500 mg/kg genistein diets reached prostate concentrations of bioavailable genistein as high as 400 and 500 nmol/L compared with 6 to 30 nmol/L and 60 to 300 nmol/L in

Table 2. Incidence of prostate tumorigenesis in TRAMP mice fed various botanical compounds

Diet	*n*	Phenotype, *n* (%)					
		Noncancer			Cancer		
		Normal	Hyperplasia	PIN	WDC	MDC	PDC
Casein	22	0	1 (4.5%)	2 (9%)	14 (63.6%)	0	5 (22.9%)
Pure 3	20	0	2 (10%)	8 (40%)*	8 (40%)	0	2 (10%)
Pure 4	18	0	8 (44.5%)†	4 (22.2%)	2 (11.1%)†	0	4 (22.2%)
Pure 7	19	0	0	11 (57.8%)†	5 (26.3%)*	0	3 (15.9%)
H-Casein	20	0	0	5 (25%)	10 (50%)	0	5 (25%)
H-Crude 7	21	0	0	15 (71.5%)*	2 (9.5%)*	0	4 (19%)

NOTE: Male TRAMP mice were started on diets at weaning and sacrificed at 5 mo. Pure 3 = curcumin + resveratrol + EGCG; Pure 4 = apigenin + baicalein + genistein + quercetin; Pure 7 = Pure 3 + Pure 4; Crude 7 = soy + sencha leaves + turmeric + yucca roots + saw palmetto + chamomile flowers + gingko; H-mice were heterozygous for estrogen receptor α. Noncancer was defined as normal, hyperplasia, and PIN. Cancer was defined as WDC, MDC, and PDC. χ^2 Test was performed to compare each treatment diet relative to casein control diet within each tumor stage.

*$P < 0.05$.

†$P < 0.01$.

serum, respectively. These values are in the range of the effect of genistein on prostate cancer cell growth inhibition and *Gli1* mRNA reduction in TRAMP-C2 cells, which were significant, starting at 100 nmol/L. Thus, dietary concentrations may be adequate to reach at least partial Hedgehog inhibition because of the ability of prostates to concentrate some compounds.

The four compounds, which inhibited Hedgehog signaling in both cell assays (genistein, curcumin, EGCG, and resveratrol), are potentially cheaper and safer alternatives to cyclopamine. All of these compounds have also been consumed in human diets for thousands of years and many are taken in dietary supplements today. There has only been a limited number of reproductive safety studies conducted, which would shed light on the potential teratogenicity of those compounds that one might hypothesize because of their cyclopamine-like effects. Genistein did not cause any fetal malformations when fed to pregnant rats up to 1,000 mg/kg/d (47). Resveratrol has been reported to act as an antiteratogenic compound (48), as has curcumin (49). Feeding pregnant rats diets supplemented with 14,000 ppm EGCG during organogenesis was nontoxic to dams or fetuses (50). Presumably, the fetus is protected from the Hedgehog-inhibitory effects of these compounds in some unknown manner.

We also used tomatidine, which is related structurally to cyclopamine, as a classic negative control in our assays (see Supplementary Fig. S7), but as reported by others, tomatidine will begin to inhibit Shh signaling at doses above 10 μmol/L (see Fig. 1C in ref. 6), albeit the cyclopamine inhibition is much stronger and will work at ~100-fold lower concentration. We used cyclopamine as a recognized positive control for Hedgehog inhibition, and clearly, it works better than tomatidine, as shown in Supplementary Fig. S7. Whereas three of our compounds Hedgehog-inhibitory activities (curcumin, EGCG, and genistein) match the potency of cyclopamine, four of the seven compounds show dose-response profiles more similar to the IC_{50} of tomatidine. This is not to say that these four botanical compounds are nonspecific but rather that they are just not as potent as cyclopamine. The ability of these botanical compounds to function, as low-cost, easily bioavailable substitutes for cyclopamine, is a major finding of this study.

Our findings that genistein, curcumin, EGCG, and resveratrol inhibit Hedgehog signaling provide prospective available, safer, and more affordable anticancer treatments for Hedgehog-driven cancers. Additionally, they help provide a better understanding of the mechanisms by which traditional herbal medicines and dietary supplements may be working to prevent and treat cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Norman Greenberg for the TRAMP mice; Dr. Jinghua Liu, Dr. Rachel Ruhlen, Dr. Gary Johnson, Dr. Shahnawaz Khan, Byron J. Bernabe, Natalie M. Abert, Amy E. Sestric, Kathryn and Charles Parker, Leslie G. Newton, and Jennifer L. Bogener for technical assistance and helpful discussions; and Dr. P.A. Beachy for kindly providing the HEK293-ShhN cell line.

Grant Support

National Center for Complementary and Alternative Medicine grant RO1 AT002978, Fisher Institute, Missouri Soybean Merchandising Council, MU Center for Phytonutrient and Phytochemical Studies, and NIH ROI-ES 510535.

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Received 08/14/2009; revised 12/18/2009; accepted 02/18/2010.

References

1. Shenouda NS, Zhou C, Browning JD, et al. Phytoestrogens in common herbs regulate prostate cancer cell growth *in vitro*. Nutr Cancer 2004;49:200–8.
2. Denis L, Morton MS, Griffiths K. Diet and its preventive role in prostatic disease. Eur Urol 1999;35:377–87.
3. Wargovich MJ, Cunningham JE. Diet, individual responsiveness and cancer prevention. J Nutr 2003;133:2400–3S.
4. Foster BA, Gingrich JR, Kwon ED, Madias C, Greenberg NM. Characterization of prostatic epithelial cell lines derived from transgenic adenocarcinoma of the mouse prostate (TRAMP) model. Cancer Res 1997;57:3325–30.
5. Sheng T, Li C, Zhang X, et al. Activation of the hedgehog pathway in advanced prostate cancer. Mol Cancer 2004;3:29.
6. Karhadkar SS, Bova GS, Abdallah N, et al. Hedgehog signalling in prostate regeneration, neoplasia and metastasis. Nature 2004;431:707–12.
7. Sanchez P, Hernandez AM, Stecca B, et al. Inhibition of prostate cancer proliferation by interference with SONIC HEDGEHOG-GLI1 signaling. Proc Natl Acad Sci U S A 2004;101:12561–6.
8. Fan L, Pepicelli CV, Dibble CC, et al. Hedgehog signaling promotes prostate xenograft tumor growth. Endocrinology 2004;145:3961–70.
9. Olsson AY, Cooper CS. The molecular basis of prostate cancer. Br J Hosp Med (Lond) 2005;66:612–6.
10. Greenberg NM, DeMayo F, Finegold MJ, et al. Prostate cancer in a transgenic mouse. Proc Natl Acad Sci U S A 1995;92:3439–43.
11. Shenouda NS, Sakla MS, Newton LG, et al. Phytosterol *Pygeum africanum* regulates prostate cancer *in vitro* and *in vivo*. Endocrine 2007;31:72–81.
12. Shappell SB, Thomas GV, Roberts RL, et al. Prostate pathology of genetically engineered mice: definitions and classification. The consensus report from the Bar Harbor meeting of the Mouse Models of Human Cancer Consortium Prostate Pathology Committee. Cancer Res 2004;64:2270–305.
13. Sasaki H, Hui C, Nakafuku M, Kondoh H. A binding site for Gli proteins is essential for HNF-3β floor plate enhancer activity in transgenics and can respond to Shh *in vitro*. Development 1997;124:1313–22.
14. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(–ΔΔC(T)) method. Methods 2001;25:402–8.
15. Rohatgi R, Milenkovic L, Scott MP. Patched1 regulates hedgehog signaling at the primary cilium. Science 2007;317:372–6.

16. Olsen B. Nearly all cells in vertebrates and many cells in invertebrates contain primary cilia. Matrix Biol 2005;24:449–50.
17. Taipale J, Chen JK, Cooper MK, et al. Effects of oncogenic mutations in smoothened and patched can be reversed by cyclopamine. Nature 2000;406:1005–9.
18. Stecca B, Mas C, Ruiz i Altaba A. Interference with HH-GLI signaling inhibits prostate cancer. Trends Mol Med 2005;11:199–203.
19. Lipinski RJ, Hutson PR, Hannam PW, et al. Dose- and route-dependent teratogenicity, toxicity, and pharmacokinetic profiles of the hedgehog signaling antagonist cyclopamine in the mouse. Toxicol Sci 2008;104:189–97.
20. El Touny LH, Banerjee PP. Genistein induces the metastasis suppressor kangai-1 which mediates its anti-invasive effects in TRAMP cancer cells. Biochem Biophys Res Commun 2007;361:169–75.
21. Mentor-Marcel R, Lamartiniere CA, Eltoum IE, Greenberg NM, Elgavish A. Genistein in the diet reduces the incidence of poorly differentiated prostatic adenocarcinoma in transgenic mice (TRAMP). Cancer Res 2001;61:6777–82.
22. Chuu CP, Chen RY, Kokontis JM, Hiipakka RA, Liao S. Suppression of androgen receptor signaling and prostate specific antigen expression by (–)-epigallocatechin-3-gallate in different progression stages of LNCaP prostate cancer cells. Cancer Lett 2009;275:86–92.
23. Harper CE, Patel BB, Wang J, Eltoum IA, Lamartiniere CA. Epigallocatechin-3-gallate suppresses early stage, but not late stage prostate cancer in TRAMP mice: mechanisms of action. Prostate 2007;67:1576–89.
24. Sartor L, Pezzato E, Dona M, et al. Prostate carcinoma and green tea: (–)epigallocatechin-3-gallate inhibits inflammation-triggered MMP-2 activation and invasion in murine TRAMP model. Int J Cancer 2004;112:823–9.
25. Siddiqui IA, Adhami VM, Afaq F, Ahmad N, Mukhtar H. Modulation of phosphatidylinositol-3-kinase/protein kinase B- and mitogen-activated protein kinase-pathways by tea polyphenols in human prostate cancer cells. J Cell Biochem 2004;91:232–42.
26. Tam NN, Nyska A, Maronpot RR, et al. Differential attenuation of oxidative/nitrosative injuries in early prostatic neoplastic lesions in TRAMP mice by dietary antioxidants. Prostate 2006;66:57–69.
27. Harper CE, Patel BB, Wang J, Arabshahi A, Eltoum IA, Lamartiniere CA. Resveratrol suppresses prostate cancer progression in transgenic mice. Carcinogenesis 2007;28:1946–53.
28. Shukla S, MacLennan GT, Flask CA, et al. Blockade of β-catenin signaling by plant flavonoid apigenin suppresses prostate carcinogenesis in TRAMP mice. Cancer Res 2007;67:6925–35.
29. Stecca B, Mas C, Clement V, et al. Melanomas require HEDGEHOG-GLI signaling regulated by interactions between GLI1 and the RAS-MEK/AKT pathways. Proc Natl Acad Sci U S A 2007;104:5895–900.
30. Dennler S, Andre J, Alexaki I, et al. Induction of sonic hedgehog mediators by transforming growth factor-β: Smad3-dependent activation of Gli2 and Gli1 expression *in vitro* and *in vivo*. Cancer Res 2007;67:6981–6.
31. Corcoran RB, Scott MP. Oxysterols stimulate Sonic hedgehog signal transduction and proliferation of medulloblastoma cells. Proc Natl Acad Sci U S A 2006;103:8408–13.
32. Dwyer JR, Sever N, Carlson M, Nelson SF, Beachy PA, Parhami F. Oxysterols are novel activators of the hedgehog signaling pathway in pluripotent mesenchymal cells. J Biol Chem 2007;282:8959–68.
33. Shaw G, Price AM, Ktori E, et al. Hedgehog signalling in androgen independent prostate cancer. Eur Urol 2008;54:133–43.
34. Chen M, Tanner M, Levine AC, Levina E, Ohouo P, Buttyan R. Androgenic regulation of hedgehog signaling pathway components in prostate cancer cells. Cell Cycle 2009;8:149–57.
35. Pu Y, Huang L, Prins GS. Sonic hedgehog-patched Gli signaling in the developing rat prostate gland: lobe-specific suppression by neonatal estrogens reduces ductal growth and branching. Dev Biol 2004;273:257–75.
36. Ingham PW, McMahon AP. Hedgehog signaling in animal development: paradigms and principles. Genes Dev 2001;15:3059–87.
37. Lamm ML, Catbagan WS, Laciak RJ, et al. Sonic hedgehog activates mesenchymal Gli1 expression during prostate ductal bud formation. Dev Biol 2002;249:349–66.
38. Yauch RL, Gould SE, Scales SJ, et al. A paracrine requirement for hedgehog signalling in cancer. Nature 2008;455:406–10.
39. Shaw A, Bushman W. Hedgehog signaling in the prostate. J Urol 2007;177:832–8.
40. Zhang J, Lipinski R, Shaw A, Gipp J, Bushman W. Lack of demonstrable autocrine hedgehog signaling in human prostate cancer cell lines. J Urol 2007;177:1179–85.
41. Corbit KC, Aanstad P, Singla V, Norman AR, Stainier DY, Reiter JF. Vertebrate smoothened functions at the primary cilium. Nature 2005;437:1018–21.
42. Gipp J, Gu G, Crylen C, Kasper S, Bushman W. Hedgehog pathway activity in the LADY prostate tumor model. Mol Cancer 2007;6:19.
43. Kasper S, Sheppard PC, Yan Y, et al. Development, progression, and androgen-dependence of prostate tumors in probasin-large T antigen transgenic mice: a model for prostate cancer. Lab Invest 1998;78:319–33.
44. Wakabayashi Y, Mao JH, Brown K, Girardi M, Balmain A. Promotion of Hras-induced squamous carcinomas by a polymorphic variant of the Patched gene in FVB mice. Nature 2007;445:761–5.
45. Dalu A, Haskell JF, Coward L, Lamartiniere CA. Genistein, a component of soy, inhibits the expression of the EGF and ErbB2/Neu receptors in the rat dorsolateral prostate. Prostate 1998;37:36–43.
46. Chang HC, Churchwell MI, Delclos KB, Newbold RR, Doerge DR. Mass spectrometric determination of genistein tissue distribution in diet-exposed Sprague-Dawley rats. J Nutr 2000;130:1963–70.
47. McClain RM, Wolz E, Davidovich A, Edwards J, Bausch J. Reproductive safety studies with genistein in rats. Food Chem Toxicol 2007;45:1319–32.
48. Jang JY, Park D, Shin S, et al. Antiteratogenic effect of resveratrol in mice exposed *in utero* to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Eur J Pharmacol 2008;591:280–3.
49. Chainani-Wu N. Safety and anti-inflammatory activity of curcumin: a component of tumeric (*Curcuma longa*). J Altern Complement Med 2003;9:161–8.
50. Isbrucker RA, Edwards JA, Wolz E, Davidovich A, Bausch J. Safety studies on epigallocatechin gallate (EGCG) preparations. Part 3: teratogenicity and reproductive toxicity studies in rats. Food Chem Toxicol 2006;44:651–61.