Common Botanical Compounds Inhibit the Hedgehog Signaling Pathway in Prostate Cancer

Anna Ślusarz1,6, Nader S. Shenouda1,6, Mary S. Sakla2,6, Sara K. Drenkhahn1,6, Acharan S. Narula7, Ruth S. MacDonald6,8, Cynthia L. Besch-Williford3,6, and Dennis B. Lubahn1,4,5,6

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 vitro in prostatic carcinoma cell lines. Our experiments, 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 IC50 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 suggest 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.

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: 1Department of Biochemistry, 2Genetics Area Program, 3Department of Animal Sciences, 4Department of Child Health, and 5MU Center for Phytonutrient and Phytochemical Studies, University of Missouri, Columbia, Missouri; 6Narula Research, Chapel Hill, North Carolina; and 7Department 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
©2010 American Association for Cancer Research.
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-3/β (JHU-73 pGL3B/8XgliBS-luc-5'-GACACACCCAA-3'; ref. 13).

The purified compounds used in tissue culture experiments were obtained from the following suppliers: apigenin (LC Laboratories), baicalin (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 ∆∆Ct 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 N2 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 vitro studies were analyzed with the χ2 test, comparing each treatment versus control diet for each cancer.
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 IC50 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 cyclopamin > 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 IC50 of <1 μmol/L, resveratrol was the most potent, followed by apigenin, baicalein, and cyclopamin. 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 IC50 values for Gli1 mRNA reduction were lower than the IC50 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 cyclopamin and genistein. 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...

---

**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. IC50 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.
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 NH2-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, baicalin, 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. S2A), with an IC50 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 twice 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.
plus EGCG were used together in Pure 3 Diet. The flavonones/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...
human prostate cancer specimens and metastases showed elevated Hedgehog pathway activity. These studies found that cyclopamine, an alkaloid isolated from *Californium veratum*, was able to inhibit human prostate cancer cell proliferation *in vitro* and 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 pharmacological 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/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

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

<table>
<thead>
<tr>
<th>Diet</th>
<th>n</th>
<th>Phenotype, n (%)</th>
<th>Noncancer</th>
<th>Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>22</td>
<td>3 (13.6%)</td>
<td>19 (86.4%)</td>
<td></td>
</tr>
<tr>
<td>Pure 3</td>
<td>20</td>
<td>10 (50%)</td>
<td>10 (50%)</td>
<td></td>
</tr>
<tr>
<td>Pure 4</td>
<td>18</td>
<td>12 (66.7%)</td>
<td>6 (33.3%)</td>
<td></td>
</tr>
<tr>
<td>Pure 7</td>
<td>19</td>
<td>11 (57.8%)</td>
<td>8 (42.2%)</td>
<td></td>
</tr>
</tbody>
</table>

†P < 0.05. ††P < 0.001. †††P < 0.01.

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. χ² Test was performed to compare cancer versus noncancer incidence for each treatment diet versus casein control diet.
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), indicating that 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

<table>
<thead>
<tr>
<th>Diet</th>
<th>n</th>
<th>Phenotype, n (%)</th>
<th>Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Normal</td>
<td>Hyperplasia</td>
</tr>
<tr>
<td>Casein</td>
<td>22</td>
<td>0</td>
<td>1 (4.5%)</td>
</tr>
<tr>
<td>Pure 3</td>
<td>20</td>
<td>0</td>
<td>2 (10%)</td>
</tr>
<tr>
<td>Pure 4</td>
<td>18</td>
<td>0</td>
<td>8 (44.5%)</td>
</tr>
<tr>
<td>Pure 7</td>
<td>19</td>
<td>0</td>
<td>11 (57.8%)</td>
</tr>
<tr>
<td>H-Casein</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>H-Crude</td>
<td>21</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

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. χ² 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 non-toxic 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 IC₅₀ 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 biouvaiable 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 R01 AT002978, Fisher Institute, Missouri Soybean Merchandising Council, MU Center for Phytonutrient and Phytochemical Studies, and NIH R01-ES 51035.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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

References


Cancer Res; 70(8) April 15, 2010
Cancer Research
Common Botanical Compounds Inhibit the Hedgehog Signaling Pathway in Prostate Cancer

Anna Slusarz, Nader S. Shenouda, Mary S. Sakla, et al.

Cancer Res 2010;70:3382-3390.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/70/8/3382

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2010/04/15/70.8.3382.DC1

Cited articles
This article cites 50 articles, 16 of which you can access for free at:
http://cancerres.aacrjournals.org/content/70/8/3382.full.html#ref-list-1

Citing articles
This article has been cited by 9 HighWire-hosted articles. Access the articles at:
/content/70/8/3382.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.