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Research Article

Cancer Prevention Research

A Phase 2 Cancer Chemoprevention Biomarker Trial of Isoflavone G-2535 (Genistein) in Presurgical Bladder Cancer Patients

Edward Messing[1], Jason R. Gee[2], Daniel R. Saltzstein[6], KyungMann Kim[2], Anthony diSant'Agnese[2], Jill Kolesar[2], Linda Harris[2], Adrienne Faerber[2], Thomas Havighurst[2], Jay M. Young[5,†], Mitchell Efros[7], Robert H. Getzenberg[8], Marcia A. Wheeler[10], Joseph Tangrea[9], Howard Parnes[9], Margaret House[9], J. Erik Busby[3], Raymond Hohl[4], and Howard Bailey[2]

Abstract

The soy compound genistein has been observed preclinically to inhibit bladder cancer growth with one potential mechanism being the inhibition of epidermal growth factor receptor phosphorylation (p-EGFR). A phase 2 randomized, placebo-controlled trial investigated whether daily, oral genistein (300 or 600 mg/d as the purified soy extract G-2535) for 14 to 21 days before surgery alters molecular pathways in bladder epithelial tissue in 59 subjects diagnosed with urothelial bladder cancer (median age, 71 years). G-2535 treatment was well tolerated; observed toxicities were primarily mild to moderate gastrointestinal or metabolic and usually not attributed to study drug. Genistein was detected in plasma and urine of subjects receiving G-2535 at concentrations greater than placebo subjects' but were not dose-dependent. Reduction in bladder cancer tissue p-EGFR staining between the placebo arm and the combined genistein arms was significant at the protocol-specified significance level of 0.10 ($P = 0.07$). This difference was most prominent when comparing the 300-mg group with placebo ($P = 0.015$), but there was no significant reduction in p-EGFR staining between the 600-mg group and placebo. No difference in normal bladder epithelium p-EGFR staining was observed between treatment groups. No significant differences in tumor tissue staining between treatment groups were observed for COX-2, Ki-67, activated caspase-3, Akt, p-Akt, mitogen-activated protein kinase (MAPK), or p-MAPK. No significant differences in urinary survivin or BLCA-4 levels between treatment groups were observed. Genistein displayed a possible bimodal effect (more effective at the lower dose) on bladder cancer tissue EGFR phosphorylation that should be evaluated further, possibly in combination with other agents. *Cancer Prev Res; 5(4); 621–30. ©2012 AACR.*

Authors' Affiliations: [1]University of Rochester Medical Center, Rochester, New York; [2]University of Wisconsin Carbone Cancer Center, Madison, Wisconsin; [3]University of Alabama-Birmingham, Birmingham, Alabama; [4]University of Iowa, Iowa City, Iowa; [5]South Orange County Medical Research Center, Tustin, California; [6]Urology San Antonio Research, San Antonio, Texas; [7]AccuMed Research Associates LLC, Garden City, New York; [8]Johns Hopkins University School of Medicine, Baltimore, MD; [9]Division of Cancer Prevention, National Cancer Institute, NIH, Bethesda, Maryland; and [10]Yale University, New Haven, Connecticut

Note: Current affiliation for A. Faerber: University of Wisconsin School of Pharmacy, Madison, Wisconsin.

Current address for J.R. Gee: Institute of Urology, Lahey Clinic Medical Center, Burlington, MA.

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[†]Deceased.

Corresponding Author: Howard Bailey, University of Wisconsin Carbone Cancer Center, Box 6164 Clinical Science Center, 600 Highland Avenue, Madison, WI 53792. Phone: 608-263-8600; Fax: 608-263-8613; E-mail: hhb@medicine.wisc.edu

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Introduction

Urothelial bladder cancer is the fifth most commonly diagnosed noncutaneous malignancy in the United States (1) and the second most prevalent malignancy in middle aged and elderly males (2). The clinical course is characterized by frequent occurrences and recurrences throughout the urothelium following initial management of non–muscle-invasive bladder cancers (3) and by extremely aggressive treatment of cancers that are more advanced or invade into the muscularis propria of the bladder. Because of this, it is the most expensive cancer to treat over the lifetime of a patient (4). Urothelial cancer is dichotomized by tumor grade; low grade represents slightly more than 50% of all newly diagnosed malignancies and high grade slightly less than 50% (2, 5). Many molecular changes characterize low- and high-grade disease, but in reality, patients often develop low- and high-grade lesions simultaneously and recurrences often differ in grade from prior tumors (6). Although more commonly associated with high-grade disease, altered expression of epidermal growth factor receptor (EGFR) is

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found in both low- and high-grade bladder cancer (7). Other molecular features also coexist in both tumor grades (8).

Urothelial cancer is a particularly appropriate target for preventive strategies, due to its biologic and clinical behavior, as well as its histologic and molecular characteristics. Unfortunately, there is minimal evidence of any advantage in tertiary chemoprevention trials using a variety of agents that are well tolerated by elderly patients (9–11). Although study design may be a factor in this lack of efficacy (9), it is possible that specifically targeted agents fail because redundant molecular pathways in the tumor make it refractory to such approaches. It was hypothesized that a natural agent with a high probability of excellent tolerance affecting many molecular pathways would be more effective.

The isoflavone genistein, a component of soy, has receptor tyrosine kinase–inhibiting activities (12) as well as phytoestrogenic effects (13). As a result, it inhibits the growth of estrogen receptor (ER)-positive human breast cancer (MCF-7) cells *in vitro* and *in vivo* (14). Genistein has also been shown in a number of cancer cell line studies to inhibit downstream modulation of genes involved in cell-cycle growth, apoptosis, angiogenesis, invasion, and motility, as well as adhesion, through a number of signaling pathways, including those involving EGF and protein kinase B (Akt; refs. 15–20). There is also *in vitro* evidence for inhibition of EGFR-mediated activity through direct competition with ATP for tyrosine kinase (K_i = 14 μmol/L) in A431 cells, but not serine or threonine kinases (21, 22).

Epidemiologic data indicate that populations with high dietary soy intake have lower incidences of bladder cancer than those following more Western diets (23, 24). Genistein has shown anti-urothelial cancer activity *in vitro* (25, 26); reported mechanisms include inhibition of cyclin B expression, induction of apoptosis, development of G_2–M cell-cycle arrest, inhibition of EGFR activity and EGF-mediated responses such as proliferation and cell motility (26), and modulation of COX-2 expression and activity (27–29). VEGF induction of COX-2 activity is inhibited by genistein in endothelial cells, another critical target for tumor growth (30). Importantly, many markers of these molecular processes can be assessed with immunohistochemistry (IHC) in formalin-fixed, paraffin-embedded tissue.

The goal of the current study was to determine whether short-term oral ingestion of 300 or 600 mg genistein/d from the soy extract G-2535 in patients diagnosed with a bladder tumor by office cystoscopy alters molecular pathways in specimens removed at transurethral resection of bladder tumor (TURBT) or cystectomy. Changes in EGFR phosphorylation at tyrosine 1068 and downstream signaling molecules as well as Ki-67 and activated caspase-3 were measured in normal-appearing and tumor urothelium tissue sections after surgery. In addition, the influence of genistein on urine levels of survivin, an apoptotic inhibitor associated with bladder cancer and predictive of superficial bladder cancer recurrence (31, 32), and BLCA-4, a nuclear matrix protein and promising bladder cancer marker (33), was assessed in voided urine during and at the completion of therapy.

Materials and Methods

Subject eligibility and recruitment

Men and women were recruited by research personnel from urology clinics at participating institutions (University of Wisconsin, Madison, WI; University of Rochester Medical School, Rochester, NY; University of Alabama-Birmingham, Birmingham, AL; University of Iowa, Iowa City, IA; South Orange County Medical Research Center, Tustin, CA; Urology San Antonio Research, San Antonio, TX; and AccuMed Research Associates LLC, Garden City, NY) in the University of Wisconsin Chemoprevention Consortium. Before determining eligibility, potential participants underwent informed consent per federal, state, and local institutional standards. Eligibility criteria included: age ≥18 years old; evidence of an initial or recurrent primary bladder tumor by cystoscopy with no evidence of distant metastases; diagnostic cystoscopy within 60 days of study enrollment and at least 45 days after bladder treatment with intravesicular agents; planned subsequent TURBT or cystectomy (partial or complete); Eastern Cooperative Oncology Group (ECOG) performance status 0 or 1; for women of childbearing potential, a negative pregnancy test; for men, agreement to use adequate contraception before study entry and during study duration; no concomitant thyroid or nonsteroidal anti-inflammatory drugs (81 mg aspirin/d allowed); no concomitant use of soy- or genistein-containing supplements; no history of pelvic irradiation; and normal organ function. Organ function parameters included the following: in marrow, white blood cells ≥3,000 mm^3, platelets ≥100,000 mm^3, and hemoglobin ≥10 g/dL; in liver, bilirubin ≤1.4 mg/dL and aspartate aminotransferase ≤3 times normal; in renal, creatinine ≤2.0 mg/dL; in metabolic functions, serum calcium ≤10.2 mg/dL and amylase ≤3 times normal; in electrolytes, sodium ≥125 and ≤155 mmol/L, potassium ≥3.2 and ≤6 mmol/L, chloride ≥85 and ≤114 mmol/L, and CO_2 ≥11 mEQ/dL; and in thyroid, thyroid-stimulating hormone within 1.3 times the upper range of normal and normal thyroxine (T_4).

Trial design

Participants meeting the eligibility criteria were randomized (1:1:1) to one of three arms: G-2535, one capsule taken orally twice a day, for a dose of 300 mg genistein/d; G-2535, two capsules taken orally twice a day, for a dose of 600 mg genistein/d; or placebo, one or two capsules taken orally twice a day for 14 to 21 days until the day before planned TURBT or cystectomy, or up to 30 days if surgery was delayed. Randomization was based on permuted blocks of size 6, stratified by clinical suspicion into non–muscle-invasive (low malignant potential or low-grade or high-grade, stage Ta, Tis, T1) versus muscle-invasive (stage T2+). Participants had safety laboratories (eligibility laboratory work plus serum lipase, T_4, and thyroid-stimulating hormone levels) done at baseline, after 1 week of study therapy,

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and on the morning of surgery. Urine for biomarkers survivin and BLCA-4 was collected at baseline, after 1 week of study therapy, and on the morning of surgery. Blood and urine for genistein and daidzein concentrations were collected at baseline, after 1 week of study therapy, and on the morning of surgery.

The primary objective of the study was to compare tumor tissue EGFR phosphorylation between treatment groups. Secondary objectives included assessment of EGFR, Ki-67, activated caspase-3, Akt, p-Akt, mitogen-activated protein kinase (MAPK), phosphorylated-MAPK (p-MAPK), and COX-2 by IHC in tumor tissues; assessment of tumor EGFR, urine survivin and BLCA-4 levels; plasma and urine genistein and daidzein concentrations; and safety and tolerance between treatment groups.

Study agent

G-2535 capsules and matching placebo capsules were manufactured by The Solae Company [Solae; formerly Protein Technologies International, Inc. (PTI)]. The active pharmaceutical ingredient, G-2535, is a purified soy extract containing ≥97% total unconjugated isoflavones and composed primarily of genistein and daidzein in a 2:1 ratio (genistein:daidzein). Each blue gelatin capsule contained enough drug substance to deliver 150 mg genistein and 75 mg daidzein. Bottles containing 60 G-2535 capsules or matching placebo capsules were distributed to the clinical research sites by McKesson Health Solutions (now Fisher BioServices, DCP Repository). Subjects randomized to the 600 mg/d dose received 2 bottles and those randomized to the 300 mg/d dose received 1 bottle. The placebo group of subjects also received either 1 or 2 bottles of placebo capsules to avoid potential bias.

Genistein doses of 300 and 600 mg/d were based on the observed pharmacokinetics and safety of the similar soy extract PTI G-2535 in prior NIH-supported and Division of Cancer Prevention, National Cancer Institute–sponsored human studies (34, 35), including one in patients with prostate cancer (36). Both doses were projected to result in urinary concentrations of genistein above the 18 to 37 μmol/L needed to slow EGF-induced proliferation of human bladder cancer cell lines in culture or to inhibit EGF-induced bladder cancer cell motility in chemotaxis assays (26). In addition, ingestion of 600-mg genistein in the form of PTI G-2535 resulted in plasma concentrations of 10 to 20 μmol/L after 12 hours, which inhibited protein tyrosine phosphorylation in peripheral lymphocytes for more than 24 hours (34).

Collection of blood (plasma) and urine for concentrations of genistein and daidzein

Before first study drug administration, at 12 hours postdose on day 8, and on the morning of surgery, 10 mL of whole blood was drawn into a heparinized tube, centrifuged for 10 minutes, with plasma aliquoted into two Nunc tubes, and frozen at −70°C before transport and eventual analysis at the UWCCC Analytical Laboratory. Urine was collected at the time of blood collection for plasma concentrations (before first study drug administration, 12 hours postdose on day 8, and on the morning of surgery). The time of the last void and the time urine was collected with total volume were recorded; four 2-mL aliquots of urine were placed into 3.6 mL Nunc tubes, stored immediately at 4°C, and frozen at −70°C within 1 to 4 hours.

Genistein and daidzein concentrations were evaluated in plasma and urine samples as previously described (37). Briefly, plasma and urine samples were extracted with methyl *t*-butyl ether. Analysis was by reverse-phase high-performance liquid chromatography (HPLC) with ultraviolet detection at 260 nm; the mobile phase consisted of 75% 0.05 mol/L ammonium formate/25% acetonitrile. All standard curves had an r^2 greater than 0.995. The lower limit of quantitation was 3.9 ng/mL for genistein and 3.1 ng/mL for daidzein. Genistein interday variability over 2 months was 16.6% for high standard ($n = 4$) and 1.6% for low standard ($n = 4$) in both matrices. Recovery of genistein from plasma was more than 90% based on comparison with H_2O standards. Daidzein plasma interday variability over 2 months was 9% for high standard ($n = 6$) and 22% for low standard ($n = 4$); and urine interday variability over 11 days was 9% for high standard ($n = 5$) and 7% for low standard ($n = 5$). Recovery of daidzein from plasma was approximately 60% based on comparison with H_2O standards.

Urine collection for survivin

Urine by clean catch was obtained before drug administration at baseline, at 12 hours postdose on day 8, and presurgery, at the end of the study; 4 mL was immediately stored at 4°C, then aliquoted into 1.7 mL eppendorf tubes within 1 to 4 hours, and frozen at −70° C. Survivin was assayed in urine by the laboratory of Dr. Robert Weiss (Yale University, New Haven, CT) using the R&D Duoset IC human total survivin ELISA (R&D DYC647) and assayed in triplicate after centrifugation and dilution of the supernatant (2 parts buffer to 1 part urine). Each microplate assayed included a standard calibration curve prepared in duplicate. Samples and control urines were assayed in triplicate, and average absorbance values used. Control samples included urine from a high-stage, high-grade patient with bladder cancer (male) collected at the time of cystectomy, and urine from a 58-year-old female with no history of bladder cancer.

Urine collection for BLCA-4

Urine by clean catch was obtained before first drug administration at baseline, at 12 hours postdose on day 8, and the morning presurgery, at the end of the study; 15 mL was immediately stored at 4°C, aliquoted into 50 mL conical tubes within 1 to 4 hours, and frozen at −70°C. Assays were conducted in the laboratory of R.H. Getzenberg at the Johns Hopkins University School of Medicine, Baltimore, MD. BLCA-4 in urine was evaluated using an adaptation of a protocol that has previously been described (38). One hundred microliters of urine was plated into a 96-well Maxisorp (Nunc 442404) plate in duplicate. The plates were

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then sealed with adhesive film and incubated overnight shaking at 300 rpm on a IKA MTS 2/4 microtiter plate shaker. The following morning, the plates were washed 3 times with 200 μL of freshly prepared 0.05% Tween-20 in PBS using a Beckman Coulter MW96 plate washer. The plates were then blocked with 200 μL of 1% bovine serum albumin (BSA), 1% dry milk, and 0.05% Tween-20 in PBS, per well, for 30 minutes at room temperature with shaking. After the blocking step, the washing step was then repeated. One hundred microliters of BLCA-4 monoclonal antibody (QCB/BioSource–Lot #0106193) diluted 1:50 in 1% BSA, 1% dry milk, and 0.05% Tween-20 in PBS was then added and incubated at room temperature for 2 hours. After the incubation, the plates were washed again and then 100 μL of goat anti-mouse secondary antibody (1 mg/mL), diluted 1:2,500 in 1% BSA, 1% dry milk, and 0.05% Tween-20 in PBS (IgG) that was human serum absorbed and peroxidase labeled (Kirkegaard & Perry Laboratories, Inc.; #074-1806, Lot #070775) was added. After a 2-hour incubation, the plates were washed again and developed with 100 μL of SureBlue TMB microwell peroxidase substrate (Kirkegaard & Perry Laboratories, Inc; #52-00-03). The absorbance was read at 630 nm with the BMG LabTech PeraStar Plate Reader. Data were collected using the MARS Data Analysis Software Ver1.20 R2 and then exported to Microsoft Excel where data points were corrected for background.

Tissue

Paraffin blocks or 10 unstained slides of normal-appearing or tumor tissue from the TURBT or cystectomy were obtained from each subject. Tissue biomarkers [total and phosphorylated EGFR (p-EGFR), Ki-67, activated caspase-3, Akt, p-Akt, MAPK, p-MAPK, and COX-2] were evaluated from malignant urothelium and normal-appearing urothelium both remote and neighboring the tumor in the Immunocytochemistry Laboratory of the Department of Pathology and Laboratory Medicine at the University of Rochester Medical Center. Immunostaining was done via DAKO Cytomation EnVision Plus system-HRP (Dako Corporation), a polymer-based proprietary system, or standard Streptavidin-HRP (horseradish peroxidase) procedure with 3-amino-9-ethylcarbamide (AEC) as the chromogen (HRP, Dako). Immunohistochemical scoring (IHS) was based on the German immunoreactive score; this method has been shown to approximate data generated from Image Analysis–based scoring systems (39). The IHS was calculated by combining an estimate of the percentage of immunoreactive cells (quantity score) with an estimate of the staining intensity (staining intensity score), as follows: 0, no staining; 1, 1%–10% of cells stained; 2, 11%–50% of cells stained; 3, 51%–80% of cells stained; and 4, 81%–100% of cells stained. Staining intensity was rated on a scale of 0 to 3, with 0, negative; 1, weak; 2, moderate; and 3, strong. The IHS was determined by multiplying quantity and staining intensity scores to produce scores ranging from 0–4, 6, 8, 9, and 12. An IHS score of 9–12 was considered strongly immunoreactive; 5–8, moderate; 1–4, weak; and 0, negative. Sections in which the staining could not be well characterized were considered equivocal.

The rabbit polyclonal anti-p-EGFR (Tyr 1068) antibody (Cell Signaling Technology) was selected for the primary immunohistochemical endpoint based on recognition of the activated conformation of EGFR with the phosphorylated C-terminal tyrosine (40). The antibody was optimized for IHC using positive and negative controls, given that the basal layer of the epidermis has been shown to be the best single positive control (41). In addition, a control tissue microarray containing 23 different normal tissues and neoplasms was used in the workup. The tyramide-catalyzed signal amplification system, GenPoint (K0620, Dako), was used to generate an optimal immunostaining signal. The DAKO Autostainer Universal Staining System was used for all staining, under stringently controlled conditions. Positive control standards were used for each run. In addition, 2 primary antibodies to nonphosphorylated EGFR (mouse monoclonals H11 from DAKO and 31G7 from Zymed) were worked up in a similar manner, as well as the DAKO EGFR PharmDX Clinical Diagnostic Kit with rigid and extensive quality control procedures for automated staining. The other immunohistochemical assays for Ki-67, activated caspase-3, Akt, MAPK, p-MAPK, COX-2, and survivin were validated using standard antibodies.

Statistical analyses

For sample size estimation/justification, the primary study endpoint of EGFR phosphorylation measured by IHS was treated as a continuous variable. Power was computed using a 2-sample Student *t* test to test IHS differences in posttreatment tissue specimens between placebo and G-2535 groups at a 2-sided significance level of 0.1. A total sample size of 36 to 93 was required to ensure a power of 0.85 for a range 0.6–1.0 of the effect size, that is, the difference in mean IHS between the placebo and G-2535–treated groups combined divided by the SD of the IHS assumed to be common. Assuming 10% random dropouts, 60 subjects (20 each in placebo, 300, and 600 mg genistein/d groups) were planned for enrollment to ensure a total of 54 subjects for primary comparison, giving at least power of 0.85 to detect the effect size of 0.8 or greater. If the rate of random dropouts was 20%, leaving a total of 48 subjects for primary comparison, the study would have at least power of 0.82 to detect the effect size of 0.8 or greater. Because of the small number of promising chemopreventive agents, it was felt important to have high power, albeit a higher false-positive probability than usual. The primary comparison between placebo and G-2535 was done using 2-sample Wilcoxon rank-sum test at a 0.1 level of significance as specified in the protocol for testing the differences in IHS in posttreatment tissue specimens between the placebo and G-2535 groups. Secondary endpoints were examined also using 2-sample Wilcoxon rank-sum test. No adjustments were made for multiple endpoints. Safety data described using NCI CTCAE version 3.0 were summarized descriptively as proportions and compared between the placebo and G-2535–treated groups using nonparametric

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tests such as Fisher's exact test or Kruskal–Wallis test. A dose–response relation was assessed using one degree of freedom analysis of covariance or Jonckheere–Terpstra test depending on the outcome.

Results

Subjects

Eighty-two subjects who signed local Institutional Review Board–approved informed consent were screened for the study from 7 sites between June 2006 and June 2008; 72 were male and 10 were female. Twenty-two of these were screen failures. Of the 60 remaining subjects whose median age was 71 years (mean, 70; range, 46–97), including 8 women and 52 men, 51 completed the study and one randomized subject dropped out of the study before receiving drug. Subjects were predominantly white, non-Hispanic (98%), and with an ECOG performance status of 0 (88%) or 1 (12%). Forty-eight (80%) of the tumors were non–muscle-invasive and 12 subjects (20%) had muscle-invasive bladder cancers (4 in each treatment group). All recorded subject characteristics appeared consistent with randomization among the 3 groups except for an imbalance in baseline weight (and body mass index) in male subjects between arms (placebo and 300 mg groups had lighter/smaller body mass index than the 600-mg group; $P < 0.05$). Baseline subject characteristics are summarized in Table 1.

Table 1. Baseline patient and disease characteristics

Treatment group	Placebo	300 mg	600 mg
Number of patients	20	20	20
Age, y			
Mean ± SD	72.0 ± 12.7	68.6 ± 9.2	68.7 ± 9.2
Median	74	68.5	68.5
Range	46–97	51–83	50–81
Sex			
Male:female	15:5	19:1	18:2
Race			
White, non-Hispanic	20	20	19
Hispanic or Latino	0	0	1
ECOG			
0	14	17	19
1	3	3	1
Weight,[a] kg			
Mean ± SD	78.3 ± 20.1	80.6 ± 14.1	90.5 ± 11.0
Range	47.7–134.9	52.6–111.4	70.7–112.3
Body mass index[b]			
Mean	27.0 ± 7.2	26.3 ± 4.4	29.5 ± 3.3
Range	16.7–44.0	18.7–35.4	24.4–39.05

[a]*P* value for males only (placebo vs. 600 mg) = 0.008; *P* value (300 mg vs. 600 mg) = 0.02.
[b]*P* value for males only (placebo vs. 600 mg) = 0.009; *P* value (300 mg vs. 600 mg) = 0.031.

Study drug compliance was generally good. Eight (14%) subjects met the criteria for noncompliance (lack of documentation of taking >95% of doses), including 5 on the placebo arm and 3 on the treatment arms (300 mg, 1; 600 mg, 2). Of the 8 subjects who dropped out of study after receiving drug, one was due to adverse events; these included grade I hypothyroidism considered unrelated to study drug, and grade I hypoesthesia, grade II dyspepsia, and grade II esophageal pain considered possibly related to study drug.

Safety

G-2535 was well tolerated; the majority of subjects experienced no or mild side effects. Table 2 displays the worst grade of adverse event (CTCAE v3) by subject in toxicity categories in which at least one grade II or worse adverse event occurred in a non-placebo subject, as well as all categories combined (overall). Examining subjects by worst postbaseline adverse event shows 12 subjects with no adverse event, 35 with grade I (mild), 8 with grade II (moderate), 4 with grade III (severe), and 1 subject with grade IV (life-threatening). The grade IV event was grade IV paroxysmal supraventricular tachycardia occurring in a placebo subject. Metabolic abnormalities (elevations in serum amylase or lipase, slight increases in serum creatinine, or decreased serum calcium) observed in numerous subjects were not related to dose. Gastrointestinal adverse events across all treatment groups included constipation; diarrhea, nausea, and heartburn were also not dose-related. Examination of all adverse events revealed no relationship between severity or frequency and increasing dose ($P = 0.12$; Jonckheere–Terpstra test).

Comparisons of treatment groups and all gradable adverse events revealed no significant differences between the placebo and treatment groups but some trends toward the treatment groups were evident. The proportion of subjects with any adverse event in the 300-mg genistein group appeared greater than placebo group ($P = 0.058$; χ^2 test) The proportion of subjects with an adverse event in the 600-mg genistein group as compared with placebo was less evident ($P = 0.144$). Comparisons of investigator-determined relationships between adverse events and study drug (definitely, probably, possibly, unlikely, or not related) between treatment groups also revealed a possible difference between the 300-mg genistein group and placebo. The 300-mg genistein group had a stronger relationship between adverse events and study treatment than placebo subjects ($P = 0.022$; Wilcoxon rank-sum test).

G-2535 is a purified soy extract containing ≥97% total unconjugated isoflavones, composed primarily of genistein and daidzein in a 2:1 ratio (genistein:daidzein). Therefore, subjects in the lower genistein dose group received approximately 300 mg genistein/d and 150 mg daidzein/d, whereas those in the high-dose group received 600 mg genistein/d and 300 mg daidzein/d. Overall, as shown in Table 3, no significant differences were seen in genistein and daidzein

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Table 2. Percentage of subjects with worst grade of adverse event for CTCAE v3 category

CTCAE category		Grade	Placebo (%)	300 mg (%)	600 mg (%)
Overall[a]	0	None	7 (35)	2 (10)	3 (15)
	1	Mild	10 (50)	14 (70)	11 (55)
	2	Moderate	1 (5)	2 (10)	5 (25)
	3	Severe	1 (5)	2 (10)	1 (5)
	4	Life-threatening	1 (5)	0 (0)	0 (0)
Metabolic/laboratory	0	None	5 (25)	7 (35)	7 (35)
	1	Mild	13 (65)	11 (55)	11 (55)
	2	Moderate	1 (5)	0 (0)	2 (10)
	3	Severe	1 (5)	2 (10)	0 (0)
	4	Life-threatening	0 (0)	0 (0)	0 (0)
Gastrointestinal	0	None	16 (80)	17 (85)	16 (80)
	1	Mild	4 (20)	2 (10)	2 (10)
	2	Moderate	0 (0)	1 (5)	1 (5)
	3	Severe	0 (0)	0 (0)	1 (5)
	4	Life-threatening	0 (0)	0 (0)	0 (0)

NOTE: Includes categories where at least one grade II or worse event occurred in a non-placebo arm.
[a]$P = 0.12$; Jonckheere–Terpstra test.

plasma or urine concentrations at baseline between the placebo and treatment groups, although both were detected in some subjects. While consumption of soy supplements was prohibited, soy intake in the diet was allowed; plasma and urine concentrations at baseline are most likely related to dietary consumption. As a group, subjects receiving placebo had no significant differences in genistein concentration from baseline to day 8 to presurgery, suggesting that dietary consumption of soy remained relatively stable during the study. On day 8, mean ± SD plasma genistein trough levels were 1.8 ± 7.0 ng/mL in the placebo group, 23.0 ± 69.6 ng/mL in the 300-mg genistein/d group, and 11.2 ± 14.2 ng/mL in the 600-mg genistein/d group. Both the 300- and 600-mg genistein/d groups were significantly different from the placebo group, but there was no significant difference in genistein concentrations between the 2 genistein-treated groups. Median trough concentrations of genistein and daidzein reflect the finding that most subjects receiving active drug had detectable peaks of genistein and daidzein in plasma below the lower limits of quantification (3.7 ng/mL for genistein and 3.1 ng/mL for daidzein). At presurgery, plasma genistein levels were 0 in the placebo group, 22.9 ± 82.4 ng/mL in the 300-mg genistein/d group, and 3.4 ± 5.9 ng/mL in the 600-mg genistein/d group. Again, there was a significant difference between the treatment and placebo groups and no difference between the 2 genistein-treated groups. The higher mean concentrations in the 300-mg genistein/d group are attributable to one individual with baseline genistein concentrations of approximately 200 ng/mL (10 times higher than any other subject's baseline level) and slight increases in genistein levels on day 8 and

Table 3. Total genistein and daidzein concentration in plasma and urine

			Genistein, ng/mL		Daidzein, ng/mL	
Treatment group	Time point	N	Plasma	Urine	Plasma	Urine
Baseline	Placebo	17	0 ± 0 (0)	59.3 ± 98.5 (21.1)	2.2 ± 6.6 (0)	305 ± 420 (61.7)
	300 mg	18	22.6 ± 93.9 (0)	212 ± 551 (47.4)	0.6 ± 2.5 (0)	650 ± 1,610 (281.0)
	600 mg	18	0 ± 0 (0)	34.9 ± 39.0 (24.9)	24.7 ± 79 (0)	1,545 ± 5,815 (135.9)
Day 8	Placebo	15	1.8 ± 7.0 (0)	57.2 ± 116 (23.6)	3.9 ± 6.8 (0)	236 ± 258 (120.0)
	300 mg	19	23.0 ± 69.6 (0–3.7)	1,235 ± 2,128 (115.5)	240 ± 982 (0–3.1)	796 ± 1,054 (452.8)
	600 mg	18	11.2 ± 14.2 (0–3.7)	648 ± 762 (288.0)	4.3 ± 6.4 (0–3.1)	975 ± 1,121 (486.7)
Presurgery	Placebo	15	0 ± 0 (0)	34.2 ± 33.8 (23.9)	1.3 ± 3.3 (0)	177 ± 230 (72.4)
	300 mg	19	22.9 ± 82.4 (0–3.7)	848 ± 977 (507.2)	54.2 ± 219 (0–3.1)	981 ± 741 (852.9)
	600 mg	18	3.4 ± 5.9 (0–3.7)	1,055 ± 1,129 (680.0)	96.1 ± 315 (0–3.1)	1,693 ± 2,731 (1,026.5)

NOTE: Values are expressed as mean ± SD (median values).

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Table 4. Phosphorylated EGFR in normal-appearing and tumor tissue

Tissue type	Grade	Placebo, *n* (%)	300 mg, *n* (%)	600 mg, *n* (%)
		N = 9	N = 14	N = 8
Benign	Negative	2 (22)	1 (7)	2 (25)
	Weak	4 (44)	4 (29)	4 (50)
	Moderate	3 (33)	8 (57)	2 (25)
	Strong	0 (0)	1 (7)	0 (0)
		N = 15	N = 19	N = 18
Tumor[a]	Negative	0 (0)	0 (0)	0 (0)
	Weak	1 (7)	5 (26)	0 (0)
	Moderate	0 (0)	4 (21)	3 (17)
	Strong	14 (93)	10 (53)	15 (83)

[a]*P* value (placebo vs. all genistein) = 0.07; *P* value (placebo vs. 300 mg) = 0.015.

presurgery. This result is likely secondary to high dietary soy intake, which frequently results in plasma levels exceeding those from supplements (42). Urinary concentrations of both genistein and daidzein were consistently higher than plasma concentrations, as has been previously reported (43).

Tissue endpoints

Tissue endpoints are summarized in Table 4 and a representative EGFR phosphorylation IHC is shown in Fig. 1.

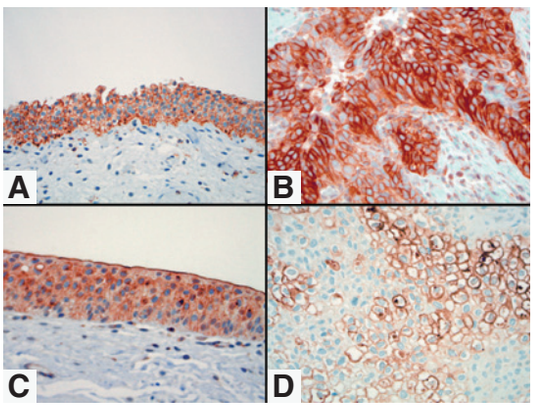

Figure 1. Representative staining for phosphorylated EGFR. A, benign urothelium from patient treated with placebo with membrane staining for phosphorylated-EGFR, immunoreactive score (IRS) moderate. B, urothelial carcinoma from patient treated with placebo with membrane staining for phosphorylated-EGFR, IRS score strong. C, benign urothelium from patient treated with 300 mg genistein with membrane staining for phosphorylated-EGFR, IRS score weak. D, urothelial carcinoma from patient treated with 300 mg genistein with membrane staining for phosphorylated-EGFR, IRS score weak. Immunohistochemical stain with hematoxylin counterstain. Original magnification, 400×.

Strong bladder tumor tissue EGFR phosphorylation, determined by IHC (3+ staining), occurred in 14 of 15 subjects in the placebo group, with lower amounts in the 2 genistein-treated groups. For the primary endpoint, comparing all genistein-treated subjects (300 and 600 mg) with placebo subjects, for the reduction (3+ staining vs. 0, 1+, or 2+ staining) in p-EGFR, a *P* value of 0.07 was observed. According to the protocol study design, in which power was designed to test the difference between the placebo and combined genistein groups at a 2-sided significance level of 0.1, this would be considered significant. In addition, examination of the treatment groups separately found a significant reduction in tumor tissue p-EGFR in subjects randomized to genistein at 300 mg/d ($P = 0.015$) but not 600 mg/d ($P = 0.44$) compared with placebo. The reduction in p-EGFR tissue staining between the placebo and 300-mg genistein/d groups was greatest in subjects with muscle-invasive bladder cancer ($P = 0.047$) than in non–muscle-invasive cancer ($P = 0.062$).

Normal bladder epithelium was also available from a majority of subjects for examination. In normal-appearing tissue, p-EGFR staining was in general less intense than in tumor tissue, especially for placebo subjects. An examination of the difference in p-EGFR between tumor and normal-appearing epithelium within each subject revealed a significantly greater proportion of placebo subjects with markedly less p-EGFR staining in normal-appearing tissue (e.g., 3+ to 1+ or 0; 2+ to 0) as compared with the 300-mg group ($P = 0.0152$) and a trend toward a significant difference when comparing placebo with all G-2535 subjects ($P = 0.063$). This was primarily due to stronger tumor p-EGFR staining in placebo subjects than in those in treatment groups. EGFR staining intensity in tumor versus normal-appearing epithelium did not differ between the treatment (together or alone) and placebo groups. Significant differences in staining were also not found between treatment groups for tumor tissue expression of COX-2 ($P = 0.32$), Ki-67 ($P = 0.35$), or activated caspase-3 ($P = 0.52$). Assessment of tumor expression of Akt ($P = 0.76$), p-Akt ($P = 0.38$), MAPK ($P = 0.41$), and p-MAPK ($P = 0.35$) also failed to reveal any differences between placebo subjects and those treated with G-2535, but similar to p-EGFR, tumor tissue from subjects in the 300-mg genistein/d group had a trend toward reduced phosphorylation of MAPK ($P = 0.15$).

Table 5. Urine survivin concentration

Treatment group	N	Baseline, pg/mL	Day 8, pg/mL	Presurgery, pg/mL
Placebo	16	16 ± 37	18 ± 33	29 ± 43
Genistein (300 mg/d)	18	71 ± 172	60 ± 175	84 ± 238
Genistein (600 mg/d)	19	47 ± 109	24 ± 41	30 ± 50

NOTE: Concentrations are expressed as mean ± SD.

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Table 6. Urine BLCA-4

Treatment group	N	Baseline (O.D.)	Day 8 (O.D.)	Presurgery (O.D.)
Placebo	17	0.46 ± 0.24	0.53 ± 0.20	0.49 ± 0.34
Genistein (300 mg/d)	20	0.52 ± 0.37	0.54 ± 0.42	0.50 ± 0.39
Genistein (600 mg/d)	19	0.55 ± 0.34	0.50 ± 0.43	0.44 ± 0.31

NOTE: Values are expressed as mean ± SD.
Abbreviation: O.D. optical density.

Survivin excretion in urine (Table 5) exhibited moderate intra- and intersubject variability. Treatment groups (300 and 600 mg genistein/d) showed a nonsignificant trend toward reduced amounts on day 8, and for the 600-mg genistein/d group, at presurgery compared with baseline. BLCA-4 excretion was unchanged across all time points and groups (Table 6).

Discussion

The doses of G-2535 chosen for this study, 150 and 300 mg genistein twice a day, produced plasma concentrations that have been shown to rapidly silence protein tyrosine phosphorylation in peripheral lymphocytes within 3 to 6 hours and maintain that effect for over 24 hours in a previous study with a similar isoflavone mixture containing lower concentrations of genistein (34). These doses also achieved urinary concentrations of unconjugated genistein that can block EGFR phosphorylation and impede EGF-induced proliferation and bladder cell motility; 18.5 to 37 μmol/L genistein has been shown to slow EGF-induced proliferation and cell motility of human bladder cancer cell lines in culture (26). These concentrations have also been shown to inhibit VEGF-induced COX-2 protein expression in human endothelial cells and in mouse macrophages *in vitro* (27). In previous clinical studies, doses of similar extracts delivering the same genistein doses were well tolerated by both men and women, including men with prostate cancer (34–36). Given the similar age range of patients with bladder cancer in this study to patients with prostate cancer treated in previous studies at 300 and 600 mg genistein/d, the same genistein doses from G-2535 were thought to be appropriate.

While this study found no significant differences in parameters other than EGFR phosphorylation, the lower dosage initiated a clear, although not statistically significant, trend of reduced expression of p-MAPK ($P = 0.145$) in tumors, primarily in subjects in whom genistein reduced EGFR phosphorylation in tumor tissue, indicating that EGFR signaling was probably affected by genistein.

G-2535 was less effective at reducing EGFR phosphorylation in normal epithelium (not shown), again indicating that EGFR activation may occur at a later stage in bladder cancer development.

This first prospective study examining the effects of genistein and other isoflavones in presurgical patients with bladder cancer was not designed to assess therapeutic or preventive effects on tumors histologically. No patients were tumor-free at surgery; such a brief exposure time was not expected to accomplish this. However, G-2535 was well tolerated and no significant adverse events were thought to be due to study drug. Since much of this study was aimed at testing tolerance and molecular response in this age and population, an effect was considered to occur if there were more responders in the treatment groups than in the placebo group. A significant ($P = 0.07$; of note, for this, initial exploratory study significance was defined as $P < 0.10$ rather than the usual $P < 0.05$) reduction of EGFR phosphorylation was seen in the genistein-treated subjects as compared with placebo with the greatest effect seen in the 300-mg genistein/d group and maximum serum levels were seen at 8 days. That a lesser effect was seen in the higher dose should not be interpreted as indicating that genistein had no effect. Evidence from other studies found that genistein can induce a bimodal response, rendering higher doses less effective than lower ones (13), as do other biologic response modifiers and natural substances (44). The possible bimodal effect (13) was hypothesized to be related to differing concentration effects upon estrogen receptor–modulated events, whether such an effect could be observed relative to EGFR phosphorylation independent or dependent of estrogen receptor–related effects is unknown. A formal dose-finding study was beyond the scope of this initial study but might be appropriate. Whether related to the lack of any observed correlation between EGFR phosphorylation inhibition and dose or genistein concentrations, blood levels of genistein were roughly as high at 300 mg/d as at 600 mg/d. The genistein and daidzein plasma concentrations assessed ≥24 hours after the last G-2535 (genistein) dose produced results consistent with the G-2535 pharmacokinetics reported by Takimoto and colleagues (45) who also observed marked interpatient variability, minimal to no dose relationship and a relatively short half-life of total and free genistein.

The most efficient study design to evaluate the effect of genistein upon normal and abnormal bladder epithelium would have had patients serve as their own controls. However, it was believed that a predrug/biopsy was so beyond the standard of care that the accrual would have been impossible—particularly, for patients with non–muscle-invasive disease (80% of subjects). Thus, an interpretation of any effect of genistein must be interpreted in the context that stratification permitted comparison of similar groups.

While our study is the first prospective clinical trial to observe a significant biologic effect of genistein in bladder epithelium, other studies have also observed evidence of the potential antineoplastic effects of genistein. An epidemiologic study in Shanghai found that individuals with higher soy product exposure by self-report had a significantly higher, rather than lower, risk of developing bladder cancer.

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While the authors did control for a variety of risk factors, only 61 bladder cancers (54 histologically confirmed) developed in 18,244 men over 10 years of follow-up (0.026% per patient-year). Even for the highest quartile, daily mean soy isoflavone ingestion was 1% to 2% of the 300-mg genistein dose in this study. The authors postulated that byproducts were formed during tofu production using soybeans soaked in possibly carcinogenic chlorinated municipal Shanghai water (46, 47). Other epidemiologic studies have reported a protective effect for dietary soy on other cancers such as breast and prostate (48–52).

Occasionally, patients with bladder cancer in the United States have diets high in soy. This was the case in 4 subjects, as determined by detectable or "therapeutic" levels of genistein in their serum at study entry. Dietary logs or questionnaires could not really determine whether this was a chronic issue or reflected large quantities ingested for brief periods immediately preceding study entry. However, if these 4 subjects are eliminated, the effects of genistein were even more dramatic on EGFR phosphorylation (not shown).

The phytoestrogenic properties of genistein were not investigated in this study (13, 14, 53). While a minority of subjects were postmenopausal women, hormonal landmarks were not recorded. Bladder cancer is primarily a disease of men (2), but ERβ has been implicated as a promoting factor for disease progression (54). Indeed, selective ER modifiers have been tested in experimental systems with some protective effects against bladder cancer (55). As stated earlier whether variable estrogenic effects by genistein could explain a possible bimodal effect in bladder epithelium is unknown. Clinical trials are ongoing with selective ER modifiers, but it is unclear whether substances with estrogenic properties fit into human bladder carcinogenesis and progression.

While genistein was, in part, selected because it had multiple effects on urothelial cancer cells in culture, *in vivo*, and in other tumor systems, the primary effect assessed in this study was inhibition of EGFR phosphorylation. The effects of 300-mg genistein/d on EGFR phosphorylation were more dramatic than the 600-mg/d dose; and those who had a response tended toward having reduced downstream signaling (e.g., MAPK phosphorylation). That these secondary effects did not reach statistical significance is perhaps more reflective of study design and numbers of subjects than lack of effect. In addition, effects on other pertinent downstream events such as proliferation (determined by Ki-67 expression), apoptosis (caspase-3 expression), and apoptosis-inhibiting markers such as survivin, were not seen, which may indicate more molecular redundancy in bladder cancer than lack of an effect of genistein *per se*. However, it is unlikely that this agent or oral soy products, when used alone, would have successful preventive or therapeutic properties. To be clinically active, such products would probably need to be combined with other treatments.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interests were disclosed.

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Cancer Prevention Research

A Phase 2 Cancer Chemoprevention Biomarker Trial of Isoflavone G-2535 (Genistein) in Presurgical Bladder Cancer Patients

Edward Messing, Jason R. Gee, Daniel R. Saltzstein, et al.

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