Phase I Pharmacokinetic and Pharmacodynamic Analysis of Unconjugated Soy Isoflavones Administered to Individuals with Cancer

Chris H. Takimoto,[3] Kira Glover,[1] Xiaoke Huang,[1] Steven A. Hayes,[1] Lilia Gallot,[1] Mary Quinn,[4] Borko D. Jovanovic,[2] Alla Shapiro,[4] Leticia Hernandez,[3] Andrew Goetz,[3,4] Victor Llorens,[3] Ronald Lieberman,[5] James A. Crowell,[5] Brett A. Poisson,[1] and Raymond C. Bergan[1]

[1]Division of Hematology/Oncology, Department of Medicine and [2]Department of Preventive Medicine, Northwestern University Medical School and the Robert H. Lurie Cancer Center of Northwestern University, Chicago, Illinois; [3]Division of Medical Oncology, Department of Medicine, University of Texas Health Science Center at San Antonio, San Antonio, Texas; and [4]Medicine and [5]Chemoprevention Branches, National Cancer Institute, NIH, Bethesda, Maryland

Abstract

Preclinical studies suggest that the isoflavone genistein may have prostate cancer chemopreventive activity. Genistein has been shown to alter cellular levels of protein-tyrosine phosphorylation and is present at high levels in soy. This study was designed to measure the pharmacokinetic parameters of two different preparations of unconjugated soy isoflavones, PTI G-2535 and PTI G-4660 (which contain 43% and 90% genistein, respectively), in human subjects with cancer, to evaluate toxicity and obtain pilot data on *in vivo* effects on protein-tyrosine phosphorylation. Cohorts of four patients were given single doses of each preparation; each dose was separated by 1 week. Sequential cohorts received genistein at 2, 4, or 8 mg/kg orally. Pharmacokinetic sampling was performed after each dose, and tyrosine phosphorylation was measured in proteins extracted from peripheral blood mononuclear cells. One of 13 patients treated developed a treatment-related rash. No other toxicities were observed. Maximal plasma concentrations (C_{max}) ranged between 4.3 and 16.3 μM for total genistein and 0.066 and 0.17 μM for free genistein. For PTI G-2535 and PTI G-4660, half-life was 15.03 and 22.41 h, respectively, and volume of distribution was 189.9 and 653.8 liters, respectively, and there was a trend toward higher area under the concentration curve for PTI G-2535 ($P = 0.07$ at the 8 mg/kg dose). Treatment-related increases in tyrosine phosphorylation were observed in peripheral blood mononuclear cells. Oral administration of soy isoflavones gives plasma concentrations of genistein that have been associated with antimetastatic activity *in vitro*.

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Requests for reprints: Raymond C. Bergan, Olson 8524, 710 North Fairbanks, Chicago, Illinois 60611-3008. Phone: (312) 908-5284; Fax: (312) 908-5717.

Introduction

Southeast Asians, who subsist on a soy bean-based diet, experience a lower incidence of metastatic prostate cancer than do those in Western countries (1–3). However, some studies suggest that the incidence of primary, organ-confined, prostate cancer may be similar in Eastern and Western populations (3, 4). Within a few generations, migrants to the West experience an increase in prostate cancer, approaching rates seen in the West. Epidemiological studies, therefore, support the notion that the metastatic behavior of prostate cancer may be amenable to pharmacological manipulation, that there is a significant clinical benefit to doing so, and that dietary and/or lifestyle factors may be causal.

Through a series of investigations, driven by epidemiological evidence, we have shown that genistein increases adhesion of human prostate cells by increasing formation of focal adhesion complexes and that it does so in a time- and concentration-dependent fashion (5–7). This action by genistein functionally antagonizes the first step in metastasis formation. Importantly, we demonstrated efficacy at concentrations as low as 1–10 nM. This is significant because blood concentrations of free genistein (*i.e.*, nonconjugated genistein) in those who subsist on a soy-based diet range between 3 and 19 nM; blood concentrations are 1–2 logs lower in non-soy consumers (8).

In addition to antimetastatic effects, a relatively large number of potential cancer chemopreventive mechanisms have been ascribed to genistein, with some of the more common mechanisms involving growth inhibition, induction of apoptosis, estrogenic activity, and antioxidant activity (7, 9–11). However, the spectrum of the pleiotropic effects of genistein is best illustrated by considering gene array-based investigations. Gene array technology can be used to identify potential drug targets and has recently been used to show that genistein does in fact modulate the expression of genes associated with a variety of cellular processes (12–14). However, most mechanistic studies have used concentrations of genistein that were in the high micromolar range and thus of unclear physiological significance.

Before evaluating the antimetastatic potential of genistein in humans, a greater understanding of its pharmacology and pharmacodynamics in humans is needed. Prospective and dietary animal studies (15, 16), as well as human dietary studies (8, 17, 18), have provided important information about the pharmacokinetics of genistein. Taken together, these studies show that increased oral consumption of soy-derived genistein is

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associated with higher blood concentrations of genistein, that this association may be linear, and that the majority of genistein is conjugated, presumably through a first pass effect in the liver. Prospective dose-escalation pharmacokinetic studies of genistein in normal volunteers, in both men and women, have been reported recently (19, 20). In parallel with those investigations, we sought to characterize the pharmacokinetics of genistein in an older cohort of men. Because cancer chemopreventive agents typically require administration for extended periods of time before realization of clinical benefits, it is important to evaluate the pharmacology of those agents in the target cohort of interest (21). In addition, we performed pilot investigations to evaluate the potential ability of genistein to alter protein-tyrosine phosphorylation in humans. Prior studies have shown that high genistein concentrations inhibit protein-tyrosine kinase activity, whereas at lower concentrations, inhibition of protein-tyrosine phosphatases appears to predominate (7, 22).

Materials and Methods

Patient Selection. Patients were entered onto an Institutional Review Board-approved protocol. Patients were eligible if they were at least 18 years old, had pathological evidence of cancer, had documented metastasis, had failed standard therapy, and were exhibiting disease progression at the time of protocol entry. Patients must have been off all forms of active therapy for at least 1 month before study entry. For patients with prostate cancer who were on a LHRH agonist, therapy with the LHRH agonist was continued to maintain castrate levels of testosterone, as is standard practice. Patients must have had an Eastern Cooperative Oncology Group performance status of 0 or 1, a life expectancy of >3 months, and intact liver (bilirubin < 2.0 mg/dl, transaminases < 3× the upper limit of normal), bone marrow (hemoglobin > 8.0 g/dl, platelets > 100,000/mm^3, and absolute neutrophil count > 1000/mm^3) and renal (creatinine < 2.0 mg/dl) function. Patients with a history of deep venous thrombosis within the past year (or on anticoagulation for such) were excluded, as were patients with known soy allergy, patients who were pregnant or breast feeding, patients with a history of breast cancer, or patients on estrogen therapy (including oral contraceptives).

Drug Administration and Toxicity Assessment. Two different preparations of unconjugated isoflavones were evaluated, PTI G-4660 and PTI G-2535. Both were manufactured under Good Manufacturing Practices guidelines by Protein Technology International (PTI, St. Louis, MO), and formulated into gelatin capsules by University Pharmaceuticals of Maryland Inc. (Baltimore, MD). Individual formulations were analyzed separately for composition by University Pharmaceuticals of Maryland, as well as by Sigma-Aldrich Inc. (St. Louis, MO). The composition of PTI G-2535 was 43% genistein, 21% daidzein, and 3% glycitein. The composition of PTI G-4660 was 90% genistein, 9% daidzein, and 1% glycitein. For each preparation, no residual protein was detected; individual capsules contained 150 mg of genistein. Preparations from a single manufacturing lot were used throughout the course of the study and provided by the National Cancer Institute.

All participants were counseled by a dietician, given a list of "forbidden foods" (*i.e.*, high soy/genistein foods), and asked to refrain from consumption of soy/genistein supplements while on study. Participants were admitted to the General Clinical Research Center of Northwestern University or the Research Unit of the National Naval Medical Center for pharmacokinetic studies and fed a low-soy diet throughout the course of the study. All meals were given at specified times during the course of study. In a sequential fashion, patients were accrued in cohorts of four onto each of three dose levels of genistein: 2; 4; and 8 mg/kg. Each patient was given a single dose of each of two separate formulations of unconjugated isoflavones. There was a 1-week interval between doses to allow for washout. Within a given dose level, the order in which individual formulations were administered to an individual patient was determined by central randomization. Patients were required to fast for 2 h before drug dosing. Drug was administered orally along with 8 ounces of water.

After drug administration, patients were monitored for clinical toxicity during their 24 h of hospitalization and then monitored daily for 2 days in the outpatient clinic, and finally monitored at the 1-month post-drug treatment time point. Clinical chemistry profiles were assessed at baseline, 1 week after each dose, and 1 month after the last dose. Clinical disease response was not formally evaluated.

Pharmacokinetic Sampling. Venous blood samples for pharmacokinetic monitoring were collected into heparin-containing tubes at the following times: before treatment and after drug ingestion at 10, 20, 30, 45, 60, 90, and 120 min and at 3, 4, 5, 6, 8, 12, 15, 24, 32, 48 and 72 h after dosing. Blood samples were kept on ice until centrifugation at 4°C, and the resultant plasma samples were stored at −80°C until analysis.

Pharmacokinetic Analysis. Plasma concentrations of total and free genistein and daidzein were measured as initially described by Supko and Phillips (16), with modifications by Thomas *et al.* (23). Briefly, 10 μl of 1 mM 4-hydroxybenzophenone in DMSO internal standard solution were added to each 1 ml of plasma and transferred to a glass test tube. Each sample was extracted with 6 ml of tert-butyl methyl ether and shaken vigorously on a rotating shaker for 30 min. After centrifugation for 10 min at 200 × *g*, the upper organic layer was transferred to a fresh glass tube and evaporated to dryness under a flow of compressed air while incubated at 50°C. Samples were redissolved in high-pressure liquid chromatography mobile phase consisting of 73:27 (v/v) 0.2 M ammonium formate (pH 4.0)/acetonitrile.

Sample separation was performed on a Waters Alliance high-pressure liquid chromatography system (Waters Corp., Milford, MA), using a Nova-Pak C8 3.9 × 150-mm reverse-phase analytical column (Waters Corp.) and a Nova-Pak C8 guard column (Waters Corp.). The injection volume was 100 μl, and an isocratic mobile phase was used with detection based on UV absorbance at 260 nm using a Waters 996 diode-array detector. Analytical standards of free genistein and free daidzein were linear over a concentration range of 5000 nM, with a lower limit of quantitation of 20 nM. Under these conditions, the retention time was 8.8 ± 0.6 min for free genistein, 4.3 min for daidzein, and 12.9 min for the 4-hydroxybenzophenone internal standard.

Total plasma genistein and daidzein were measured by mixing 250 μl of plasma with 0.5 ml of a freshly prepared enzyme solution consisting of 0.2 M ammonium acetate (pH 4.0), 85.2 mM ascorbic acid, and 500 μl of β-glucuronidase/sulfatase from *Helix pomatia* (Sigma Chemicals, St. Louis, MO). Samples were incubated overnight for 15–18 h at 37°C and cooled to room temperature. Then, 10 μl of internal standard solution were added, and samples were extracted in the same fashion as described for the free genistein and daidzein assay described above. Genistein and daidzein were obtained from Sigma Chemicals and used to prepare standard solutions.

Plasma pharmacokinetics were characterized using noncompartmental analytical methods as implemented in WinNon-

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Lin Standard, Version 3.1 (Pharsight Corp., Mountain View, CA). Elimination rate constants were estimated by linear regression of the terminal log linear portion of the concentration-time curves. Terminal half-lives ($T_{1/2}$) were calculated by dividing 0.693 by the elimination rate constants. The AUC was calculated using the linear trapezoidal rule with extrapolation to infinity (24). Systemic clearance (CL/F) was determined by dividing the dose by the AUC, and the apparent volume of distribution during the terminal elimination phase (Vz/F) was calculated from the formula: $Vz/F = dose/(\lambda z \times AUC_{0-\infty})$. The MRT[6] was calculated from the formula: MRT = AUMC/AUC, where AUMC is the area under the first moment curve. Pharmacokinetic parameters were summarized as means and SDs, except for half-lives, for which harmonic means and pseudostandard deviations were used (25). Covariate influence on individual pharmacokinetic parameters was examined by visually inspecting bivariate scatterplot graphs as implemented in S-Plus 2000 (Mathsoft Info), and correlations were examined further by linear regression analysis. Ideal body weight for men (all subjects were men) was calculated according to following formula, as reported previously (26): Ideal body weight (kg) = 51.65 + (1.85 × [the number of inches above 60]).

Analysis of Tyrosine Phosphorylation of Proteins from PBMNCs. Blood for isolation of PBMNCs was collected into 30-ml syringes containing preservative-free heparin at baseline, 6 h, and 24 h. Samples were processed immediately after collection. PBMNCs were isolated by density gradient centrifugation, as described previously (27, 28), subjected to hypotonic lysis to remove residual RBCs, washed in PBS three times, snap frozen, and stored at −70°C. Protein-tyrosine phosphorylation was measured in a batch fashion by Western blot, as described previously (9). Briefly, ice-cold lysis buffer [50 mM Tris (pH 7.5), 0.1% SDS, 0.5% deoxycholic acid, 150 mM NaCl, and 1% NP40 containing the protease inhibitors aprotinin (10 μg/ml), leupeptin (20 μg/ml), and phenylmethylsulfonyl fluoride (1 mM), plus 1 mM orthovanadate to inhibit protein-tyrosine phosphatase activity] was added to cell pellets immediately upon thawing. Equal amounts of protein from clarified lysates were separated on an 8% SDS polyacrylamide gel under reducing and denaturing conditions, and transferred onto 0.45 μm nitrocellulose (Schleicher and Schuell, Keene, NH) in a wet transfer cell. Blots were blocked with fraction V BSA (Sigma Chemicals), probed with anti-phosphotyrosine monoclonal antibody (clone 4G10; Upstate Biotechnology, Lake Placid, NY), followed by horseradish peroxidase-conjugated secondary antibody, and visualized with the Renaissance chemiluminescent system (Amersham, Arlington Heights, IL). All Western blots were repeated once, at a separate time.

A number of quality control measures were performed, and samples that did not meet them were excluded from analysis. Samples were excluded from analysis if they were not processed within 1 h of collection. They were also excluded if the protein concentration of the time 0, 6 h, and 24 h time point samples differed by more than 20% for a given patient and a given formulation. Blots were also stained with amido-black to evaluate protein loading. Finally, each Western blot included one lane of cell lysate from a previously characterized stock of cell lysate from human PC3-M prostate cells, as a positive control.

The density of individual bands on resultant phosphotyrosine Western blots was determined by scanning gels on a Molecular Dynamics Densitometer SI and using ImageQuant software (Molecular Dynamics) to obtain band density. Density of phosphotyrosine bands was then normalized for degree of protein loading. This was done by determining the density of a sample of 10 randomly selected protein bands on amido-black-stained blots. These 10 individual band densities were then summed, thus giving an average measure of protein loading that could be used for normalization purposes. Only samples run on the same gel were subjected to comparison.

[6] The abbreviations used are: MRT, mean residence time; PBMNC, peripheral blood mononuclear cell.

Results

Patient Characteristics and Clinical Outcomes. Between October 1999 and June 2000, 13 patients were accrued onto the study, and 12 completed it. Median performance status was 0, median age was 67 years (range, 40–86 years), 11 (85%) participants had prostate cancer, and 2 (15%) had colon cancer (Table 1). There were 13 men and no women, reflecting the large number of patients with prostate cancer; 38% of participants were African American, and 8% were Asian.

Of 13 patients accrued, all 13 (100%) received their first dose of drug; 12 patients (92%) received both doses. One patient, who had colon cancer, voluntarily withdrew from the study. Toxicity was observed in only one patient, and it consisted of a maculo-papular erythemic rash on the extremities and face, appearing after the first dose, worsening after the second dose, and resolving spontaneously thereafter.

Pharmacokinetic Studies. Pharmacokinetic samples were collected from 11 patients during cycles 1 and 2. One patient only received one cycle of genistein 90% preparation at 8 mg/kg, and no kinetic data were available for another patient treated at 8 mg/kg. One additional patient treated at 4 mg/kg had no total drug sample data due to insufficient amounts of plasma. Total plasma genistein and daidzein represent the sum of the free and conjugated drug forms circulating in plasma. Sufficient concentration *versus* time data were available for detailed pharmacokinetic analysis of total genistein and daidzein levels in plasma, whereas low plasma concentrations of free drug precluded a detailed kinetic analysis of these drug species. For the 43% preparation of genistein, C_{max} (Fig. 2*A*) and AUC (Fig. 2*B*) increased with increasing dose level. However, the 90% preparation did not demonstrate a proportional

Table 1 Patient characteristics

Characteristic	No	%
Age (yrs)		
Median	67	
Range	40–86	
Race		
Caucasian	7	54
African American	5	38
Asian	1	8
ECOG[a] performance status		
Median	0	
0	9	69
1	4	31
Sex		
Male	13	100
Female	0	0
Type of cancer		
Prostate	11	85
Colon	2	15

[a] ECOG, Eastern Cooperative Oncology Group.

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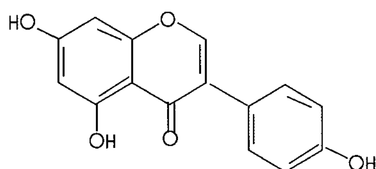

Fig. 1. Structure of genistein.

increase in C_{max} (Fig. 2*C*) or AUC (Fig. 2*D*) over this same dose range, consistent with non-dose-proportional drug kinetics. Although interpatient variability was high, the 90% preparation appeared to generate lower total drug plasma AUC values, particularly with higher genistein doses (Tables 2 and 3). At the 8 mg/kg dose level, AUC values were 221.2 ± 3.3 and 112.0 ± 96.6 μM·h for 43% and 90% preparations, respectively, and differences approached statistical significance ($P = 0.07$). Interestingly, although participants were extensively counseled regarding avoidance of soy-containing foods, baseline concentrations of total genistein were above the level of detection in 5 of the 12 individuals tested (42%). The average mean ± SD total genistein concentration in these five individuals was 0.33 ± 0.16 μM.

The estimated total genistein pharmacokinetic parameters are summarized by dose level in Tables 2 and 3 for the 43% and 90% preparations, respectively. The mean ± SD apparent clearance (CL/F) for total genistein was 8.86 ± 6.39 and 14.75 ± 17.38 liters/h for the 43% and 90% preparations, respectively, and the volume of distribution during the terminal elimination phase (Vz/F) was 189.9 ± 124.3 and 653.8 ± 830.8 liters for the 43% and 90% preparations, respectively. The median apparent time to maximum concentration (T_{max}) was 6.0 h (range, 3.0–24 h) and 4.5 h (range, 2.0–24.0 h), and the mean ± SD apparent half-life ($T_{1/2}$; harmonic mean ± pseudostandard deviation) was 15.03 ± 2.61 and 22.41 ± 10.40 h for the 43% and 90% preparations, respectively.

The pharmacokinetic parameters for daidzein are summarized in Tables 4 and 5. Total plasma daidzein kinetics were similar in general to genistein, with a trend toward higher total plasma drug concentrations with the 43% genistein preparation that approached statistical significance at the 8 mg/kg dose level ($P = 0.06$). The concentrations of free genistein were considerably lower than that of total genistein (Table 6). For the 43% and 90% preparations, respectively, the median apparent T_{max} of free genistein was 3.0 h (2.0–24 h) and 3.0 h (0.5–10.0 h), and mean C_{max} values for the 2, 4, and 8 mg/kg dose levels ranged from 66.4 to 154.8 and 53.2 to 116.9 nM.

The impact of individual patient covariates on the clearance of total plasma genistein was examined after dosing with the 43% preparation. Covariates analyzed included age, weight, ethnicity, histological tumor type, history of prior chemother-

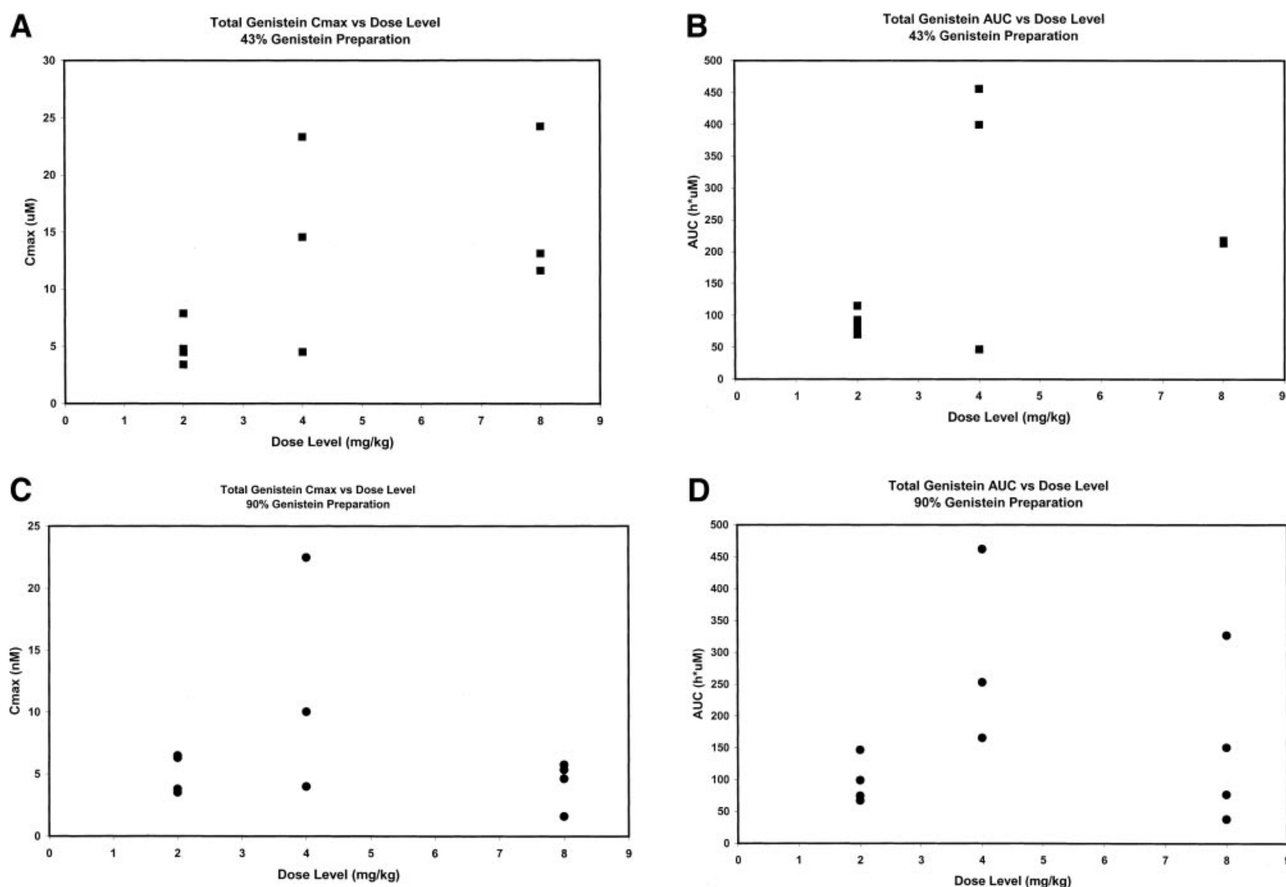

Fig. 2. Pharmacokinetic parameters associated with 43% and 90% preparations of genistein. In *A* and *C,* C_{max} (maximum concentration) values are depicted, and in *B* and *D,* AUC (area under the curve) values are depicted, as a function of genistein dose (2, 4, and 8 mg/kg). In *A* and *B,* the 43% preparation of genistein was administered; in *C* and *D,* the 90% preparation of genistein was administered.

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Table 2 Total plasma genistein pharmacokinetic parameters after dosing with 43% genistein (means ± SD)

Subject no.	Tablets taken	Dose level (mg/kg)	No. of patients	T_{max}[a,b] (h)	C_{max} (μM)	$AUC_{0-\infty}$ (μM·h)	Apparent $T_{1/2}$[c] (h)	CL/F (liters/h)	V_Z/F (liters)	MRT (h)
1	1	2	4	6.0 (4.5–6.0)	5.12 (1.91)	91.1 (20.3)	14.48 (2.76)	6.31 (1.35)	141.1 (64.4)	19.91 (2.53)
2	1									
3	1									
4	1									
5	3	4	3	4.5 (3–4.5)	14.12 (9.43)	301.4 (223.2)	16.83 (3.46)	10.63 (11.66)	230.7 (215.0)	23.17 (7.80)
6	3									
7	2									
8	4	8	3	8.0 (4.5–24)	16.34 (6.90)	221.2 (3.3)[d]	13.87 (0.51)[d]	11.28 (1.61)[d]	226.4 (40.6)[d]	18.10 (0.98)[d]
9	5									
10	5									
	Totals		10	6.0 (3–24)			15.03 (2.61)	8.86 (6.39)	189.9 (124.3)	20.60 (4.69)

[a] T_{max}, time of maximal plasma concentration; C_{max}, maximal plasma concentration; $AUC_{0-\infty}$, area under the concentration-time curve extrapolated to infinity; CL/F, apparent clearance; $T_{1/2}$, apparent terminal half-life of elimination; V_Z/F, apparent volume of distribution during the terminal elimination phase.
[b] Median and range.
[c] Harmonic means and pseudostandard deviations.
[d] Unable to estimate terminal elimination in one patient, $n = 2$.

apy, and history of prior radiation therapy. Interestingly, no correlation was observed between ideal body weight and drug clearance (coefficient of determination, $R^2 = 0.13$; Fig. 3).

Protein Phosphotyrosine Analysis. There were 13 different sets of samples available for analysis, from 9 different patients. A set of samples consists of blood cells collected from time 0 (pretreatment baseline) and 6 and 24 h after treatment for a given patient receiving a given preparation of genistein. Depicted in Fig. 4 are resultant phosphotyrosine Western blots, as well as associated amido-black blots (a measure of total protein loading). Changes in tyrosine phosphorylation in a number of protein bands were evident. However, significant patient-to-patient variability was also evident. Because changes in the level of tyrosine phosphorylation of a 60-kDa protein band were particularly prominent, changes in its tyrosine phosphorylation were evaluated further. An increase in tyrosine phosphorylation was observed at the 6 h time point (*i.e.*, at a time corresponding to high concentrations of plasma genistein) in 10 of 13 sets of samples (77%) or 7 of 9 patients (78%) evaluated.

Quantitation of changes in the 60-kDa band intensity (Table 7) demonstrated that, compared with relatively low baseline values, there was a 2.8 ± 0.9-fold (mean ± SE; $n = 12$; $P = 0.03$) increase in band intensity at the 6 h time point, decreasing to 1.1 ± 0.2-fold, compared with baseline, by 24 h ($P = 0.67$). Note that the phosphotyrosine intensity of the 60-kDa band at the 0, 6, and 24 h time points corresponded to the concentration of genistein. Changes in tyrosine phosphorylation were observed with all genistein dose levels and preparations examined. Whereas the small sample size precludes rigorous statistical analysis, no apparent trends were evident with respect to differential phosphorylation as a function of preparation or dose. Furthermore, there was no significant association between change in tyrosine phosphorylation and pretreatment genistein level or type of prior therapy.

Discussion

Accrual was heavily skewed toward prostate cancer, reflecting the patient population seen by the investigators. Toxicity was minimal and consisted of a self-limiting rash in one person that

Table 3 Total plasma genistein pharmacokinetic parameters after dosing with 90% genistein (means ± SD)

Subject no.	Tablets taken	Dose level (mg/kg)	T_{max}[a,b] (h)	C_{max} (μM)	$AUC_{0-\infty}$ (μM·h)	Apparent $T_{1/2}$[c] (h)	CL/F (liters/h)	V_Z/F (liters)	MRT (h)
1	1	2	6.0 (3–8)	5.02 (1.58)	92.2 (32.3)	15.53 (2.37)	6.28 (2.00)	138.7 (31.0)	22.06 (2.32)
2	1								
3	1								
4	1								
5	3	4	4.5 (3–8)	12.18 (9.42)	205.7 (206.6)	29.48 (26.76)	5.63 (1.76)	341.4 (229.9)	58.21 (30.72)
6	3								
7	2								
8	4	8	3.0 (2–24)	4.34 (1.88)	112.0 (96.6)	30.45 (11.83)	30.04 (22.30)	1403.2 (1031.3)	45.67 (21.3)
9	5								
10	4								
11	5								
	Totals		4.5 (2–24)			22.41 (10.40)	14.75 (17.38)	653.8 (830.6)	40.52 (23.82)

[a] T_{max}, time of maximal plasma concentration; C_{max}, maximal plasma concentration; $AUC_{0-\infty}$, area under the concentration-time curve extrapolated to infinity; CL/F, apparent clearance; $T_{1/2}$, apparent terminal half-life of elimination; V_Z/F, apparent volume of distribution during the terminal elimination phase.
[b] Median and range.
[c] Harmonic means and pseudostandard deviations.

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Table 4 Total plasma daidzein pharmacokinetic parameters after dosing with 43% genistein (means ± SD)

Genistein dose level (mg/kg)	Daidzein dose level (mg/kg)	No. of patients	T_{max}[a,b] (h)	C_{max} (μM)	$AUC_{0-\infty}$ (μM·h)	Apparent $T_{1/2}$[c] (h)	CL/F (liters/h)	V_Z/F (liters)	MRT (h)
2	0.98	4	4.5 (1–48)	2.36 (1.85)	27.7 (22.0)	6.69 (2.32)[d]	25.6 (30.8)[d]	206.0 (198.6)[d]	10.37 (1.18)
4	1.95	3	4.5 (4.5–6)	9.88 (6.54)	167.9 (118.3)	9.20 (16.70)	7.42 (5.79)	100.7 (21.4)	21.98 (14.4)
8	3.91	3	8 (6–24)	10.11 (4.23)	481.3 (636.1)	7.48 (4.86)	8.16 (6.16)	85.4 (9.0)	32.9 (36.5)
Totals		10	6.0 (1–48)			7.65 (4.58)	13.7 (18.3)	134.1 (114.2)	21.7 (21.9)

[a] T_{max}, time of maximal plasma concentration; C_{max}, maximal plasma concentration; $AUC_{0-\infty}$, area under the concentration-time curve extrapolated to infinity; CL/F, apparent clearance; $T_{1/2}$, apparent terminal half-life of elimination; V_Z/F, apparent volume of distribution during the terminal elimination phase.
[b] Median and range.
[c] Harmonic means and pseudostandard deviations.
[d] Unable to estimate terminal elimination in one patient, $n = 3$.

Table 5 Total plasma daidzein pharmacokinetic parameters after dosing with 90% genistein (means ± SD)

Genistein dose level (mg/kg)	Daidzein dose level (mg/kg)	No. of patients	T_{max}[a,b] (h)	C_{max} (μM)	$AUC_{0-\infty}$ (μM·h)	Apparent $T_{1/2}$[c] (h)	CL/F (liters/h)	V_Z/F (liters)	MRT (h)
2	0.2	4	4.5 (3–8)	1.53 (0.99)	30.0 (21.3)	7.3 (10.0)	1.18 (1.64)	15.6 (12.9)	29.12 (28.47)
4	0.4	3	8 (8–24)	29.6 (42.9)	1296 (1710)[d]	33.8 (14.4)[d]	0.23 (0.30)[d]	15.3 (20.9)[d]	42.63 (2.18)[d]
8	0.8	4	6.25 (4.5–8)	1.23 (0.51)	39.3 (27.9)	8.00 (10.1)	2.5 (1.92)	58.0 (37.3)	56.55 (53.88)
Totals		11	8.0 (3–24)			9.05 (10.7)	1.52 (1.73)	32.5 (32.4)	42.79 (37.49)

[a] T_{max}, time of maximal plasma concentration; C_{max}, maximal plasma concentration; $AUC_{0-\infty}$, area under the concentration-time curve extrapolated to infinity; CL/F, apparent clearance; $T_{1/2}$, apparent terminal half-life of elimination; V_Z/F, apparent volume of distribution during the terminal elimination phase.
[b] Median and range.
[c] Harmonic means and pseudostandard deviations.
[d] Unable to estimate terminal elimination in one patient, $n = 2$.

likely represented an allergy to soy. However, because treatment time was short, additional toxicities will likely become apparent when long-term soy treatment is evaluated prospectively.

Estimates of average daily genistein consumption by soy consumers range from 0.3 to 1 mg/kg (29–41). Thus, participants in the current study were given 2–8× the maximum average dietary intake. Although participants subsisted on a red meat-based diet and were instructed regarding avoidance of soy products, 42% had baseline plasma levels of genistein that were above the level of detection. Within this group, the average baseline concentration of genistein was 0.33 μM. Interestingly, this corresponds closely to the average plasma level of genistein previously reported by Adlercreutz *et al.* (8) in normal Japanese men, which was 0.276 μM; in contrast, average levels in non-soy consumers (Finish men) were 0.006 μM. Whereas the Adlercreutz study evaluated normal volunteers, the current study evaluated subjects with cancer. Genistein is promoted through the mass media for its anticancer effects and is commercially available. Because rates of nutriceutical consumption by cancer patients are high (42, 43), it is likely that participants were taking soy supplements (either knowingly or unknowingly). Self-administration of experimental agents is an important consideration in cancer chemoprevention (44); pharmacokinetic monitoring of blood levels is thus an important part of chemoprevention studies (21). Because some putative chemopreventive dietary constituents have been shown to be harmful when studied prospectively (45), a cautionary note of the potential for harm is raised to eager investigators who overemphasize preliminary findings.

Both epidemiological (1–4) and preclinical mechanistic studies (5–7, 46) suggest that soy-associated genistein inhibits prostate cancer metastasis, and the lower limit of pharmacological efficacy seen in preclinical models was 1–10 nM (6, 7). These concentrations directly overlap with the 3–19 nM blood levels of free genistein measured in those who subsist on a soy-based diet, which in turn is 1–2 logs above the levels seen in non-soy consumers (8). The current study showed that after ingestion of twice dietary amounts of genistein, peak plasma levels of free genistein were 66–117 nM, thus supporting the

Table 6 Pharmacokinetic parameters for free plasma genistein

Genistein dose level (mg/kg)	43% Genistein preparation			90% Genistein preparation		
	No. of patients	T_{max}[a,b] (h)	C_{max}[c] (nM)	No. of patients	T_{max}[b] (h)	C_{max} (nM)
2	4	3.25 (2–24)	66.4 (46.3)	4	3.0 (0.5–10)	116.9 (116.6)
4	3	3.0 (2–4.5)	169.8 (27.5)	2	6.0 (2–10)	108.7 (116.2)
8	3	3.0 (2–3)	154.8 (96.6)	2	3.25 (2–4.5)	53.2 (42.7)

[a] T_{max}, time of maximal plasma concentration; C_{max}, maximal plasma concentration.
[b] Median and range.
[c] Means and SDs.

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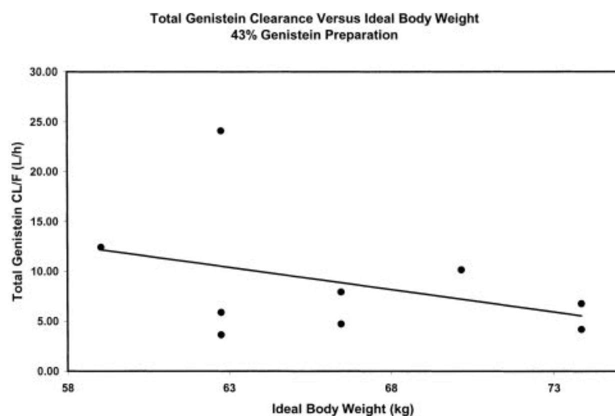

Fig. 3. Association between body weight and clearance in people treated with genistein. Clearance values as a function of ideal body weight are depicted.

notion of administration of higher doses to attain relatively rapid effects on biological behavior.

Whereas there was a linear relationship between dose and concentration for the 43% preparation, this did not appear to be the case for the 90% preparation of genistein. There was a nonsignificant trend toward increased clearance and decreased AUC and $C_{\max}$ for the 90% preparation, compared with the 43% preparation, particularly at higher doses. Similar findings were reported by Busby *et al.* (19). Elucidation of underlying mechanisms will require further study. Potential explanations include decreased first pass metabolism or interference by daidzein or other constitutions in the less pure 43% preparation, difference in volume of distribution, and/or greater saturation of absorption pathways with the 90% preparation. The half-life of genistein was higher in our study, as compared with that of Busby *et al.* (19). However, there were important differences between the two studies. Different formulations of isoflavones were used. In addition, Busby *et al.* (19) accrued normal volunteers, whereas the current study accrued an older cohort of men with comorbid conditions. Because chemopreventive agents are typically administered over extended periods of time, it is important to define pharmacokinetics in the cohort of interest (21). Older men represent a likely target cohort for a prostate cancer chemopreventive agent (44).

An important new pharmacokinetic observation was the apparent lack of association of apparent clearance of total plasma genistein with absolute body weight. This represents a preliminary finding that needs to be evaluated in expanded studies. Genistein doses were scaled to body weight, implicitly assuming greater clearance by larger individuals, a commonly adopted practice in oncology. This practice has recently come under criticism (47–49), and our data would further support such concerns. Our data suggest that absolute body weight does not predict increased clearance of total genistein, and thus, our dosing scheme may have actually inflated the interpatient variability in plasma drug concentrations. Further development of this agent should use fixed dose levels for all individuals.

Although we (7) and others (22) have shown that genistein can alter protein-tyrosine phosphorylation in cell culture systems, it was surprising to detect significant changes *in vivo* after only a single dose of drug. However, if in fact genistein were exerting significant biological effects on cancer, and because all doses significantly exceeded average dietary intake, it is not unreasonable to expect pharmacological effects throughout the dose range evaluated. Different patterns of tyrosine-phosphorylated proteins were seen in different patients, as were changes in phosphorylation with genistein treatment. This likely represents a sensitive measure of interindividual variability, including such factors as metabolizing enzymes, coadministration of other drugs and/or foods, and consumption of soy products. Interindividual variability is a common theme in pharmacotherapeutics, and its implications for cancer chemoprevention have recently been reviewed (21). In fact, significant interindividual variability in excretion of isoflavones and lignans after soy ingestion has been reported previously (50). Importantly, because tyrosine phosphorylation plays a central role in cell signaling, modulation of tyrosine phosphorylation status *in vivo* provides a measure of *in vivo* effects on intracellular signaling (46).

Whereas it is tempting to evaluate apparent differences in the intensity of baseline tyrosine phosphorylation between patients, the current study was not designed to measure such potential differences. Factors potentially responsible for observed differences relate to differences in the amount of cell-associated protein available for analysis from individual patients, as well as patient-specific differences in the number and type of circulating cells. The identity of the 60-kDa protein whose tyrosine phosphorylation is increased by genistein is

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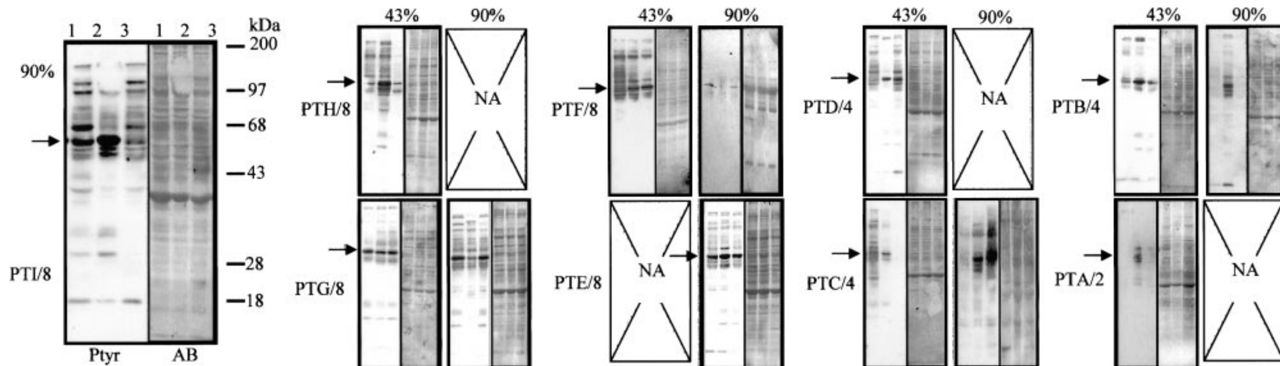

Fig. 4. Effects of oral genistein upon protein-tyrosine phosphorylation in PBMNCs. PBMNCs were collected at time 0 (baseline), 6 h (high genistein) and 24 h, after soy dosing (*Lanes 1–3*, respectively), and the resultant cytoplasmic proteins were probed for phosphotyrosine by Western blot (*Ptyr*) and then stained with amido-black (*AB*) to evaluate protein loading, as described in "Materials and Methods." Blots from individual patients (*PT*) are marked with letters (*A−I*), and the dose of genistein is indicated in mg/kg (*e.g.,* PTA/2 indicates patient A received 2 mg/kg). The associated preparation of genistein (43% or 90%) is marked *above* each set of blots. Molecular mass standards are provided in kDa, and *arrows* denote 60 kDa. *NA,* not available for analysis.

Table 7 Intensity of phosphotyrosine bands*

Patient	Genistein, (mg/kg)	Preparation	Ratio of band intensity	
			6 h	24 h
A	2	43	2.4	0.3
B	4	43	3	1.5
B	4	90	2.7	0.9
C	4	43	2.4	0.4
C	4	90	12.8	22
D	4	90	2.5	3.1
E	8	90	1.5	1.4
F	8	43	1.8	1.3
F	8	90	0.8	0.9
G	8	43	0.9	1
G	8	90	0.6	0.7
H	8	43	3.2	1
I	8	90	2.4	0.3
All		Mean	2.8	1.1
		SE	0.9	0.2

*Changes in the intensity of 60-kDa phosphotyrosine bands at 6 and 24 h after genistein treatment are shown. Presented are the ratios of band intensity for the 6 and 24 h time points, as compared with baseline. Phosphotyrosine band intensities were normalized for the level of protein loading, as described in "Materials and Methods."

currently unknown. We have implemented a Phase II study of genistein in men with prostate cancer. This study will provide the opportunity to pursue the identity of this protein and evaluate the effect of long-term genistein administration and its potential impact on long-term changes in protein-tyrosine phosphorylation. Likely candidates for the 60-kDa protein include src, lck, lyn, fyn, yes, and p62(dok).

Genistein has long been considered a nonspecific protein-tyrosine kinase inhibitor and is thus commonly used as such, albeit at concentrations of 100 μM and above (22). In contrast, we have shown that at lower concentrations, genistein increases the protein-tyrosine phosphorylation content of human prostate cells treated *in vitro*, suggesting inhibition of phosphatase activity (7). Taken together, data suggest differential effects as a function of concentration. This notion is directly supported by the current study, wherein increases in tyrosine phosphorylation were observed *in vivo*, in the face of nanomolar concentrations of free genistein. It is important to note that genistein has pleiotropic effects, and further investigation is required to evaluate the full spectrum of *in vivo* effects. Because genistein is known to have estrogenic activity, caution needs to be exercised if one is considering its use in women at risk for breast cancer.

The current study demonstrates that dietary components, namely soy, given in dietary proportions, can modulate *in vivo* cell signaling pathways. If in fact dietary factors pharmacologically modulate predisposition to cancer, as a large body of research suggests (51), it therefore stands to reason that dietary components can modulate molecular events *in vivo*. This study provides evidence of such. Currently, there is not enough information to support the development of one particular preparation of isoflavones over another. The 90% preparation seems attractive because of its greater "purity" and because many preclinical studies point to genistein as having chemopreventive activity. However, it is possible that clinical activity, if present at all, is due to combinations of components found in soy, which may or may not include genistein.

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