Soy-Derived Isoflavones Inhibit the Growth of Canine Lymphoid Cell Lines

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Abstract

Purpose: This study aimed to evaluate the *in vitro* effects of genistein, both pure genistein and a commercially available form of genistein called Genistein Combined Polysacharride (GCP), against two canine B-cell lymphoid cell lines and determine the oral bioavailability of GCP when fed to normal dogs.

Experimental Design: The *in vitro* effect of genistein and GCP was evaluated using cell proliferation and apoptotic assays. The IC_{50} of both compounds was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay and propidium idodide staining. Apoptosis was evaluated using Annexin V staining, caspase 3 and 9 staining, and DNA laddering. Cell cycle analysis and Bcl-2/Bax ratios were also examined. An initial dose escalating pharmacokinetic study was used to determine if therapeutic serum levels of genistein could be reached with oral dosing of GCP in normal dogs.

Results: The 72-hour *in vitro* IC_{50} of genistein and GCP against the GL-1 and 17-71 cells were both 10 μg/mL and 20 μg/mL, respectively. GCP led to cell death in both cell lines via apoptosis and treated cells exhibited increased Bax:Bcl-2 ratios. The serum concentrations of genistein in normal dogs given increasing oral doses of GCP did not reach the 72-hour *in vitro* IC_{50} in a dose escalation study.

Conclusions: The results of these studies support the notion that canine high-grade B-cell lymphoma may represent a relevant large animal model of human non-Hodgkin's lymphoma to investigate the utility of GCP in chemopreventive and/or treatment strategies that may serve as a prelude to human clinical lymphoma trials.

Genistein (4, 5, 7-trihydroxyisoflavone) is a readily available isoflavone found in soy-based products. Epidemiologic studies indicate that consumption of soy-containing diets is associated with a lower incidence of many human tumors (1, 2). Genistein has been identified as an inhibitor of various protein tyrosine kinases that play a role in cell growth and apoptosis, including camp-responsive element-binding protein (3), signal transducers and activators of transcription (4), members of the fork-head-related transcription factors (5), and nuclear factor κB (NF-κB; ref. 6). At pharmacologic concentrations genistein's recorded activities also include topoisomerase I and II inhibition (7), antioxidant activity (8), induced differentiation (9), and deregulation of mitochondrial membrane pore permeability (10).

Genistein's effects on various human solid cancer cell lines have been extensively studied (11). Although the precise molecular mechanisms responsible for these activities are not clearly understood, the compound can inhibit cancer cell growth (12, 13), induce apoptotic cell death with cell cycle arrest at G_2-M phase, and inhibit angiogenesis (14). Genistein causes epigenetic changes in mouse prostate (15) and up-regulates mRNA expression of the *BRCA1* tumor suppressor gene during mammary tumorigenesis (16). The compound also inhibits DNA methyltransferase and reverses the methylation status, with concomitant reexpression, of the $p^{16INK4a}$, RARb, and *MGMT* genes in human esophageal squamous cell carcinoma and prostate cell lines (17).

Genistein is also active against human lymphoid neoplasia (18, 19). Genistein induces apoptosis via mitochondrial damage in T lymphoma cells (20) and via Akt signaling in anaplastic large-cell lymphoma (21). The molecule also reduces NF-κb in T lymphoma cells via caspase-mediated cleavage of Iκβα (22). Finally, when included into a CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone) lymphoma protocol, genistein has growth modulatory effects, via G_2-M arrest and decreased NF-κb binding, in a diffuse large B-cell lymphoma xenograft setting (23).

Natural and synthetic genistein glycosides are not easily absorbed across enterocytes in humans and cats (24, 25). Genistein Combined Polysacharride (GCP), a commercially available form of genistein (26), is a complex mixture produced by fermentation of soybean extracts with a mushroom mycelia (*Ganoderma lucidum*) culture that contains approximately 40%

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Translational Relevance

Canine lymphoma has historically been considered an excellent animal model of a variant of human non-Hodkin's lymphoma called diffuse, large B-cell lymphoma. Dogs are large, long-lived animals that are evolutionarily more closely related to humans than rodents; therefore, they represent an accessible, spontaneous population of high-grade lymphomas occurring in immunocompetent animals. Based on a large body of human literature documenting the antiproliferative effects of genistein, we hypothesized that genistein would also have *in vitro* activity against canine lymphoid cell lines and perhaps *in vivo* activity in canine lymphoma patients in either chemopreventive or treatment protocols. The results presented here support our hypothesis that genistein has significant *in vitro* antiproliferative activity against two well-established canine B-cell lines, mirroring human cell line data. Additionally, we developed an extremely sensitive high-pressure liquid chromatography – based assay to detect plasma genistein in dogs fed a commercially made, highly bioavailable form of genistein called Genistein Combined Polysacharride (GCP). With this assay, we were able to show that genistein in GCP can be absorbed by canine gut enterocytes, which leads to detectable genistein plasma levels. These initial studies with GCP lay the groundwork for future studies in the setting of canine lymphoma with findings that may be directly applicable to human diffuse large B-cell lymphoma.

isoflavones. Fermentation deglycosylates soy isoflavones, producing aglycone isoflavones that are highly absorbable across the gut lumen (27). GCP has documented *in vitro* and *in vivo* effects on a wide variety of human cancers (28, 29).

Canine high-grade B-cell lymphoma, which is similar phenotypically and biologically to the most common variant of human non-Hodgkin's lymphoma called diffuse large B-cell lymphoma, is one of the most common malignant tumors of dogs (up to 25% of all cancers) and it is the most common hematopoietic tumor of the dog (83%; ref. 30). The median survival time of canine B-cell lymphoma patients treated with CHOP or L-VCAP (asparaginase, vincristine, cyclophosphamide, adriamycin, prednisone)-based protocols is 12 to 14 months, with an overall cure rate of <10% (31). Dogs are evolutionarily more closely related to humans than rodents and are large, long-lived animals; therefore, canine B-cell lymphoma represents an excellent model to investigate novel therapeutics and treatment strategies that may have direct applicability to the human disease (32).

Based on the similarity between canine B-cell lymphoma and human diffuse large B-cell lymphoma, we hypothesized that genistein would inhibit the cell growth of two well-established canine lymphoid B-cell lines. We show that genistein induces cell death via apoptosis at concentrations similar to those reported in the human literature. We also present our preliminary findings of the oral absorption of GCP in domestic dogs.

Materials and Methods

Canine cell lines. The nonadherent canine B-cell lines 17-71 and GL-1 have been previously described (33, 34). Both lines were maintained in RPMI 1640 (Mediatech, Inc.) plus 10% heat-inactivated fetal bovine serum (Mediatch, Inc.), 1% L-glutamine (Mediatech, Inc.), and 100 mg/mL primocin (InvivoGen), and grown at 37°C/5% CO_2 in a humidified atmosphere.

Genistein treatment. Genistein (Sigma) was dissolved in DMSO to make a 200 mg/mL stock solution that was added directly to the culture medium to produce various concentrations. The same volume of DMSO was added to untreated control cells, which produced a final concentration of DMSO in the range of 0.001 to 0.045%. GCP is a functional supplement developed and donated by Amino Up Chemical Co. Ltd. The detailed information of this product is provided on the website.[3] Compositional analysis of the GCP extract supplied by the manufacturer revealed that 116 ± 8.4 mg of genistein, 28.5 ± 5.4 mg of daidzein, 13.5 ± 2.6 mg of glycitein, and ~3% of insoluble polysaccharides from basidiomycetes are present per gram of GCP. GCP was dissolved in 50% ethanol as previously described (28). A stock solution of GCP was prepared that contained 200 μg/mL of genistein. The same volume of 50% ethanol was added to untreated control cells, which produced a final concentration of ethanol of 1%.

Cell proliferation assays. The 17-71 and GL-1 cells were seeded in 96-well plates at a density of 1×10^6 cells/well in a final volume of 100 mL. The cells were grown in media alone or media containing either the vehicle control or increasing concentrations of synthetic genistein or GCP (from 5 to 100 μg/mL). The culture was maintained in the CO_2 incubator for an additional 3 d. For each GCP concentration and time point, cultures were maintained in triplicate. At the end of the 24-, 48-, and 72-h time periods, 10 μL of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) labeling reagent was added [Cell Proliferation Kit 1 (MTT), Roche Applied Science] and the plates were incubated for an additional 4 h. One hundred microliters of solubilization solution were then added and the plates were incubated overnight. The formazan spectrophotometrical absorbance of the samples at 570 nm was measured the next day using a Sunrise microplate reader (Tecan Trading AG). These absorbance values were compared with values of carrier-only treated cells and standard curves generated with varying concentrations of the untreated cell lines.

Using these experiments, the 48-h IC_{50} of both pure genistein and GCP was calculated.

Apoptotic assays. Early apoptotic events were detected using an Annexin V-PE Apoptosis Detection Kit (BD Biosciences) per the manufacturer's instructions. Briefly, 1×10^6 cells were treated with the IC_{50} of GCP as described above for 72 h. After collection and washing twice with cold PBS, the cells were suspended in 1× binding buffer. 1×10^5 of the cells were transferred to another tube and either 5 μL of Annexin V-PE, 5 μL of 7-amino-actinomycin (7-AAD), or both, were added. After 15 min in the dark at room temperature, 400 μL of binding buffer were added. Flow cytometric analysis was immediately performed (FASCan; Becton-Dickinson).

Early apoptosis was also documented by examining caspase 9 and 3 activation (ApoptosisCaspGLOW Fluorescein Active Caspase-3 and 9 Staining Kits; Biovision, Inc.) per the manufacturer's instructions. Briefly, 1×10^6 cells were treated with the IC_{50} of GCP as described above for 72 h. 3×10^5 cells were stained with 1 μL of FITC-LEHD-FMK (FITC-conjugated caspase 9 inhibitor), and FITC-DEVD-FMK (FITC-conjugated caspase 3 inhibitor) and incubated at 37°C for 1 h. Treated cells were also stained with a caspase activation inhibitor supplied with the kit. The cells were washed twice with PBS and analyzed by flow cytometry (FASCan).

Late apoptosis was documented via DNA "laddering" using an apoptotic DNA kit (ApopLadder Ex; GenWay) per the manufacturer's instructions using 5×10^6 cells that were treated with the IC_{50} of GCP as described above for 72 h. The DNA was quantitated using a NanoDrop ND 1000 spectrophotometer (NanoDrop Technologies). Two micrograms of DNA and a PhiX174/Hae III DNA ladder were separated in a

[3] http://www.aminoup.co.jp

1.5% agarose gel and visualized using ethidium bromide staining and an Alpha Innotech Multi-wavelength illuminator (Alpha Innotech).

Bax and Bcl-2 staining. 1×10^6 cells were treated with the IC_{50} of GCP for 72 h. Intracellular Bax and Bcl-2 staining of GCP-treated cells was done in duplicate using a commercial permeabilizing kit per the manufacturer's instructions (Leucoperm, AbD Serotec). Briefly, after harvesting, 100 μL of fixation medium were added and incubated for 15 min at room temperature. After centrifugation and supernatant removal, 1.25 μg of anti-Bax (BD Transduction Laboratories), anti-Bcl-2 (BD Transduction Laboratories), and goat antimouse IgG FITC (Jackson ImmunoResearch) antibodies were added and incubated on ice for 30 min in the dark. One hundred microliters of permeabilization medium were then added and incubated for 30 min at room temperature. IgG FITC alone was used as a secondary control for nonspecific staining. Mean fluorescent intensities were analyzed by flow cytometry (FASCan). The Bax:Bcl-2 ratio was calculated for carrier-only and GCP-treated cells were normalized to untreated control cells using the formulas: Bax RMFI = Bax MFI/MFIC, Bcl-2 RMFI = Bcl-2MFI/MFIC, and Bax:Bcl-2 ratio = Bax RMFI/Bcl-2 RMFI, where RMFI is relative mean fluorescent intensity, MFI is mean fluorescent intensity, and MFIC is mean fluorescent intensity of control (secondary antibody only).

Cell cycle analysis. Cell cycle analysis was done using propidium iodide staining. After GCP treatment as described above for 72 h, 1×10^6 cells were washed twice with PBS/0.1% glucose and resuspended in 1 mL ice cold 70% ethanol. After centrifugation, the cells were washed with PBS and stained with 500 mL of a propidium iodide working solution that contained 17.96 mL PBS/0.1% glucose, 2 mL stock propidium iodide solution (0.5 mg/mL propidium iodide in PBS; BD Biosciences), and 4 μL DNase-free RNAse A (100 mg/mL; Qiagen). At least 10,000 events were collected by flow cytometry (FASCan).

GCP pharmacokinetic study design. Three non–specific pathogen-free, healthy, purpose-bred, intact adult male mongrel dogs purchased from a commercial vendor (Covance Research Products) and maintained by The North Carolina State University Laboratory Animal Resources (NCSU-LAR) facility were used. The dogs, weighing 31.5 kg, 27.5 kg, and 25.4 kg, were fed once daily with a mix of commercial wet and dry dog food and had *ad libitum* access to water. All procedures were approved by the Institutional Animal Care and Use Committee at North Carolina State University. The maintenance and care of the dogs complied with the guidelines set forth in the guide for the Care and Use of Laboratory Animals (NIH).

Jugular catheters were aseptically placed for blood collection the night before GCP supplementation. Pre-GCP baseline complete blood counts (CBC) and serum chemistries were collected. The dogs were fed once in the morning with an experimental diet consisting of their normal food supplemented with 0.45% GCP. Three-milliliter blood samples were collected into lithium heparin glass tubes serially at 20, 40, and 60 min, and 2, 4, 6, 8, 24, and 48 h postdosing. Immediately after collection, the plasma was isolated and frozen at -80°C. Based on previous studies (35) we did not have any reason to suspect that there would be degradation of our samples during storage. At 48 h, CBC and serum chemistries were drawn and the dogs were returned to NCSU-LAR. CBCs and serum chemistries were also collected 1 and 2 weeks post-GCP administration. One and two months later, the same three dogs were fed an experimental diet supplemented with 1.0% and 1.45% GCP, respectively, following the same procedures outlined above. The amount of GCP fed to the dogs was based on previous murine studies (28, 29).

During the study period, the dogs were monitored visually for signs of unacceptable gastrointestinal toxicity. The definition of unacceptable toxicity was ≥2 animals in a single dose group who experienced grade 3 or higher gastrointestinal toxicity refractory to standard supportive care. Blood/bone marrow and constitutional signs toxicity was unacceptable when toxicity was ≥2 animals in a single-dose group experiencing grade 3 or grade 4 hematologic toxicity and nausea/fatigue, respectively, lasting >7 d. These toxicity guidelines were based on The Veterinary Cooperative Oncology Group-Common Terminology Criteria for Adverse Events (VCOG-CTCAE), v1.0, 2004. (36).

Plasma genistein high-pressure liquid chromatograph analysis. Ca-Canine plasma samples were analyzed by high-pressure liquid chromatograph (HPLC). The assay for genistein was developed by one of the investigators (MGP), after modification from previous published methods (24, 35). Modifications were made to the assay for a different matrix (canine plasma) and to produce a method that could be done rapidly on many samples.

Genistein was dissolved in 100% methanol to create a stock solution (1 mg/mL) of genistein base. The stock solution was further diluted by the addition of 50:50 distilled water:methanol to prepare genistein fortifying solutions. Blank (control) plasma collected from untreated dogs was used to create calibration standards that ranged in concentration from 0.01 to 5.0 μg/mL. The mobile phase for the HPLC analysis consisted of distilled water (60%) and acetonitrile (40%). Trifluoroacetic acid (0.1%) was added to the mobile phase to lower the pH and improve shape of the elution peaks.

The HPLC system consisted of a quaternary solvent delivery system (Agilent 1100 series; Agilent Technologies) at a flow rate of 1 $mL.min^{-1}$, an autosampler (Agilent 1100 series autosampler; Agilent Technologies), and UV detector (Agilent Variable Wavelength Detector; Agilent 1100 series, Agilent Technologies) at a wavelength of 260 nm. The chromatograms were integrated with a computer program (Agilent Chemstation Software, Agilent 1100 series; Agilent Technologies). The analytical column was a reverse-phase, 4.6 mm × 15 cm C18 column (Zorbax SB-C18, Mac Mod) kept at a constant temperature of 40°C.

All incurred plasma samples, calibration samples, and blank (control) plasma samples were prepared in an identical manner. Solid-phase extraction cartridges (Bond Elut-CN-E; Varian) were conditioned with 1 mL of methanol followed by 1 mL of distilled water. Each plasma sample (500 μL) was added to a conditioned cartridge, followed by a wash step of 1 mL of distilled water:methanol (95:5). The drug was eluted with 1 mL of 100% methanol and collected in clean glass tubes. The tubes were heated at 40°C and evaporated under a flow of air for 20 min to yield a dry residue. Each tube was then reconstituted by the addition of 200 μL of mobile phase, vortexed briefly, and transferred to a HPLC injection vial. Fifty microliters of each sample were then injected into the HPLC system. Laboratory procedures were conducted in accordance with guidelines published by the United States Pharmacopeia.

Retention time for the peak of interest was 4.4 to 4.6 min. Calibration and blank samples were prepared for assays on each day. All calibration curves were linear with a $r^2 \geq 0.99$. Limit of quantification for genistein in canine plasma was 0.01 μg/mL, which was determined from the lowest point on a linear calibration curve that yielded acceptable precision and accuracy. Quality control samples were analyzed concurrently with the incurred and calibration samples.

Pharmacokinetic analysis. Plasma drug concentrations were plotted on linear and semilogarithmic graphs for analysis. Analysis of curves and pharmacokinetic modeling was done using a commercial pharmacokinetic program (WinNonlin Version 5.2; Pharsight Corporation). A compartmental analysis was done initially, but it was obvious that there was no consistent compartmental model that would be suitable for all dogs and doses. For some dogs, when plotting the individual plasma concentration versus time curves, there was an initial decline in concentrations to approximately 4 h, followed by a gradual increase. Because of this pattern in drug absorption, a terminal slope or terminal half-life was calculated from all of the terminal points, but resulted in an uncertain value because of poor linearity of the terminal slope (poor r^2 value for terminal slope). Because of the uncertainty of this calculation, the terminal half-life calculated from this study should be interpreted cautiously.

Compartmental rate constants could not be calculated using this data because there was not an accompanying i.v. dose; therefore, a noncompartmental analysis that does not assume any compartmental structure was used.

For the noncompartmental analysis the area under the plasma concentration versus time curve (AUC) from time 0 to the last measured

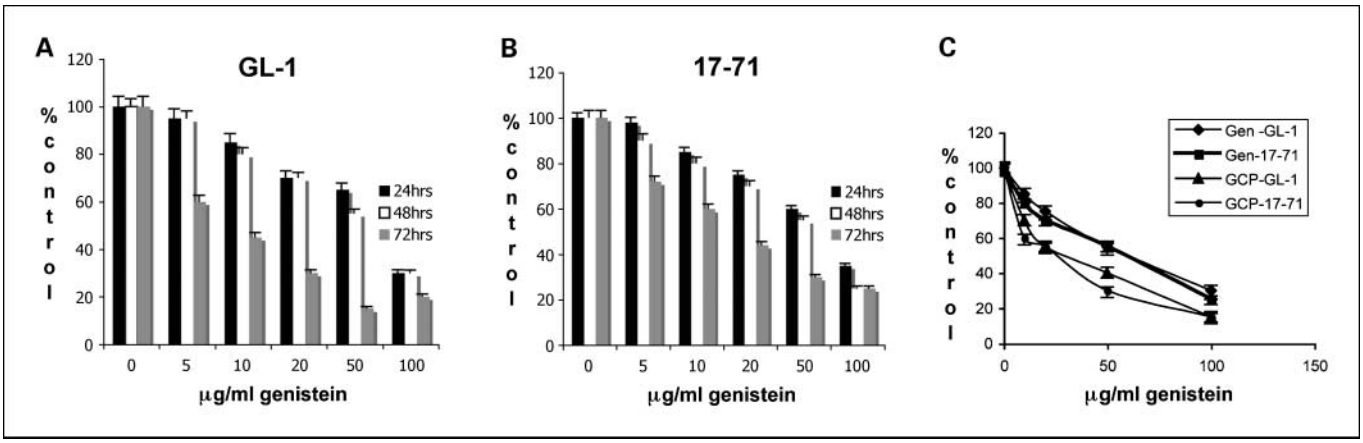

Fig. 1. Inhibition of proliferation of two canine B cell lines, (*A*) GL-1 and (*B*) 17-71, by genistein and GCP. Proliferation of both cell lines was assessed in the presence of various concentrations of either compound for 72 h in a 96-well format using a MTT cell proliferation kit. Absorbances were measured at 570 nm and expressed in relation to absorbances of control (carrier only) cells. Values are expressed as means + SD ($n = 3$). $P < 0.005$ (compared with control cells in both GL-1 and 17-71 cells). *C,* comparison of purified genistein to amounts of GCP extract with equivalent concentrations of genistein on cell growth of both GL-1 and 17-71 cells over 72 h. Cell growth was analyzed as described above.

concentration (defined by the sensitivity of the assay) was calculated using the log-linear trapezoidal method. The AUC from time 0 to infinity was calculated by adding the terminal portion of the curve, estimated from the relationship Cn/λ_Z, to the $AUC_{0\text{-}cn}$, where λ_Z is the terminal slope of the curve, and Cn is the last measured concentration point. Values for the maximum plasma concentration after dosing (C_{MAX}) and time to maximum plasma concentration (T_{MAX}) were determined. Half-lives were calculated from the terminal slope (when possible): $T^1/_2$ = ln 2.0/(terminal rate constant).

Results

Both genistein and GCP decrease the* in vitro *growth of two canine B-cell lines by affecting proliferation. This study analyzed the effect of pure genistein and GCP on the cell growth of two well-characterized canine lymphoid cell lines, GL-1 and 17-71. These cell lines were exposed to increasing concentrations of genistein (5-100 μg/mL) and their viability was followed for 72 hours using a MTT cell proliferation assay. Cell growth over the 72-hour period for the GL-1 and 17-71 cell lines treated with genistein is shown in Fig. 1A and B, respectively. The dose-response graphs are presented as percentage of control growth on the corresponding day, and each value is measured in triplicate. There was minimal growth inhibition evident in the vehicle-treated (ethanol at a final concentration of 1% or DMSO at a concentration < 0.05%) control cells. Additionally, GCP was found to be a more potent inhibitor of cell growth in both cell lines when purified genistein was compared with equivalent concentrations of genistein in the GCP extract (Fig. 1C). Based on these experiments, we calculated the 72-hour *in vitro* IC_{50} of both genistein and GCP against the GL-1 and 17-71 cells to be 10 μg/mL and 20 μg/mL, respectively. For the remainder of the experiments, only GCP was used.

***GCP induces apoptosis in GL-1 and 17-71 cells* in vitro.** Genistein induces G_2-M cell cycle arrest cytostasis and/or apoptotic cell death in a variety of human tumors (14, 37). We show a similar effect of GCP on the canine lymphoid cell lines. Flow cytometric analysis of GL-1 and 17-71 cells treated with the IC_{50} of GCP for 72 hours resulted in an increased population of cells localizing to the sub-G_0 region of the histogram (Fig. 2A and B). The percentage of cells in the sub-G_0 region in GL-1 and 17-71 cells was increased to 81% and 62%, respectively, after GCP treatment (*grey lines*). These results show that GCP seems to initiate the process of apoptosis rather than acting as a cytostatic agent (no marked increase in the G_2-M population) in the canine lymphoid cell lines, which may

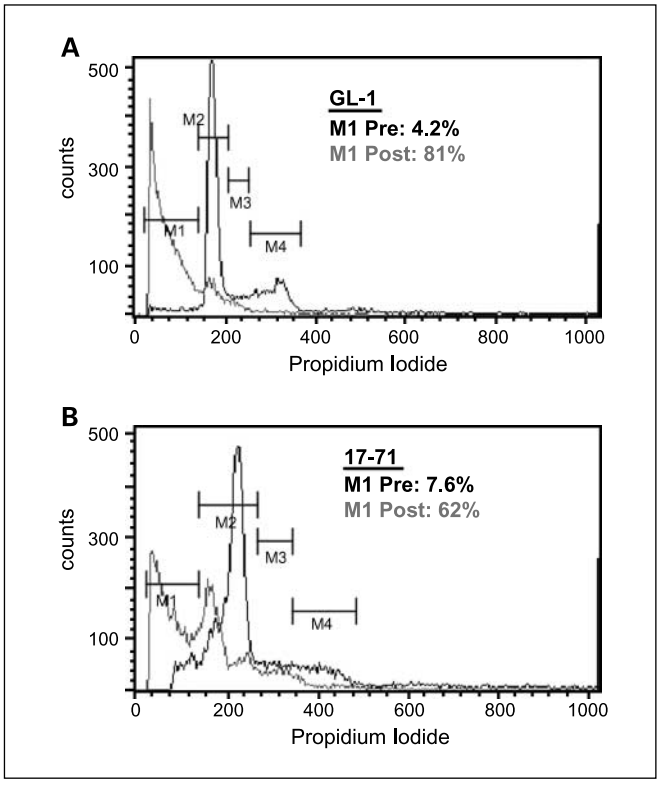

Fig. 2. GCP induces sub-G_0 localization of both (*A*) GL-1 and (*B*) 17-71 cells. Cells were exposed to the IC_{50} of GCP for 72 h. Adherent and floating cells were pooled, washed, and stained with propidium iodide as described. Cell cycle distribution was analyzed on at least 10,000 cells for each experimental condition. Data analysis was done using CellQuest cell cycle analysis software. Black tracings, pre-GCP treatment histograms; grey tracings, post-GCP treatment histograms.

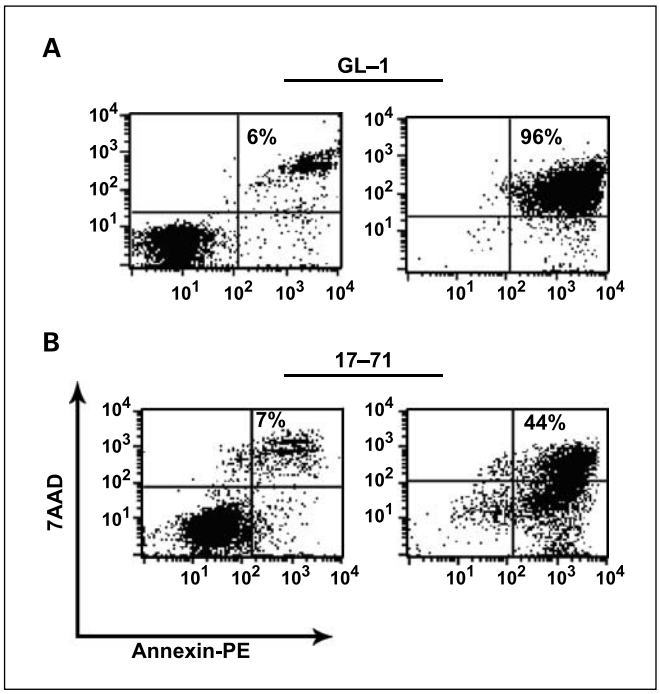

Fig. 3. GCP induces apoptosis in both (*A*) GL-1 and (*B*) 17-71 cells. Cells were exposed to the IC_{50} of GCP for 72 h. Adherent and floating cells were pooled, washed, stained with an anti-Annexin antibody and 7-AAD as described and analyzed by flow cytometry to detect early signs of apoptosis. Scatterplots were generated and analyzed using CellQuest cell cycle software. The left scatter plot for both cell lines is representative ($n = 3$) of cells treated with carrier only, whereas the right scatter plot is representative ($n = 3$) of GCP-treated cells.

account for the growth inhibition observed earlier in the cell proliferation studies.

To further document evidence of GCP-induced apoptosis, we looked for characteristic markers of early and late apoptosis in the GCP-treated cells. The translocation of membrane phospholipid phosphatidylserine from the inner to the outer leaflet of the plasma membrane, an early event in the apoptotic cascade, can be detected via staining with Annexin V. After GCP treatment for 72 hours, the cells were stained with Annexin V-PE and the vital dye 7-AAD, and analyzed by flow cytometry (Fig. 3A and B). Approximately 90% of the GCP-treated GL-1 cells stained with both Annexin V and 7-AAD, suggesting the cells had undergone apoptotic cell death within 3 days of treatment. In contrast, although approximately 84% of the 17-71 cells stained with Annexin V, only 37% were 7-AAD–positive, indicating apoptotic cell death was delayed in this cell line.

Because caspases play a central role in apoptosis, we also examined the induction of apoptosis of GCP-treated cells by examining the activation of the cytsteine proteases caspase 9 and 3. The effects of GCP on caspase activation in GL-1 and 17-71 cells is shown in Fig. 4. The percentages presented in the diagram are the percentage of caspase 9- or 3-positive cells in GCP-treated cells (*grey lines*) when compared with cells treated with carrier alone (*black lines*). In Panel A, GCP treatment for 72 hours led to caspase 9 activation in 60% and 63% of GL-1 and 17-71 cells, respectively. In Panel B, the same treatment led to caspase 3 activation in 48% and 43% of GL-1 and 17-71 cells, respectively. Addition of a caspase activation inhibitor supplied by the manufacturer returned caspase 9 and 3 activation levels to within 10% of the carrier-only controls (data not shown).

Caspase activation also activates DNases, which leads to the programmed degradation of genomic DNA into characteristic 200-bp nucleosomal fragments, an event that occurs late in the apoptotic cascade. These DNA fragments can be seen as a "laddering" effect when separated via gel electrophoresis. In Fig. 4C, lanes 4 and 7 represent GCP-treated GL-1 and 17-71 cells, respectively. No DNA laddering was seen in either untreated or carrier-only–treated GL-1 cells (*lanes 2 and 3*), whereas some minor DNA laddering can be seen in the untreated and carrier-only–treated 17-71 cells (*lanes 5 and 6*), although GCP treatment of the 17-71 cells led to the more marked laddering seen in lane 7.

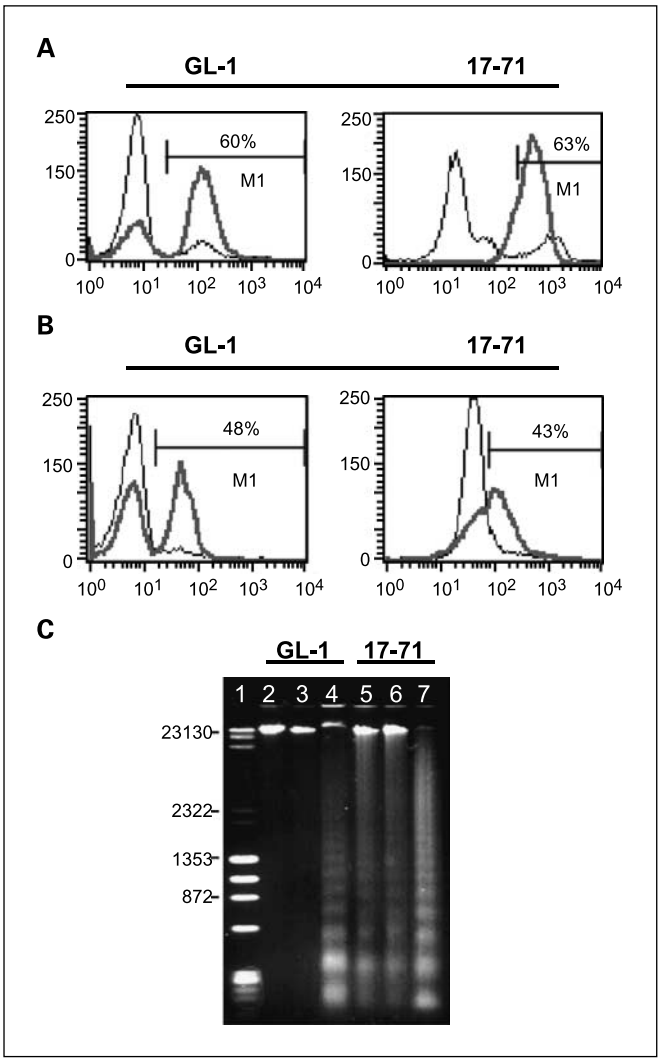

Fig. 4. GCP induces caspase activation leading to DNA degradation in both GL-1 and 17-71 cells. In *A* and *B*, cells were exposed to the IC_{50} of GCP for 72 h. Adherent and floating cells were pooled, washed, stained with an (*A*) anti-caspase 9 antibody and (*B*) an anti-caspase 3 antibody, and analyzed by flow cytometry. Histograms were generated and analyzed using CellQuest cell cycle software. In all histograms, the thin black tracings are representative ($n = 3$) of cells treated with carrier only, whereas the thick grey tracings are representative ($n = 3$) of GCP-treated cells. *C*, after exposure of GL-1 and 17-71 cells to the IC_{50} of GCP for 72 h, adherent and floating cells from both lines were harvested and washed. DNA was isolated using an apoptotic DNA kit as described and separated on a 1.5% agarose gel. Lane 1, PHIX174/Hae III DNA ladder; lanes 2 and 5, untreated cells; lanes 3 and 6, carrier-only – treated cells; lanes 4 and 7, GCP-treated cells.

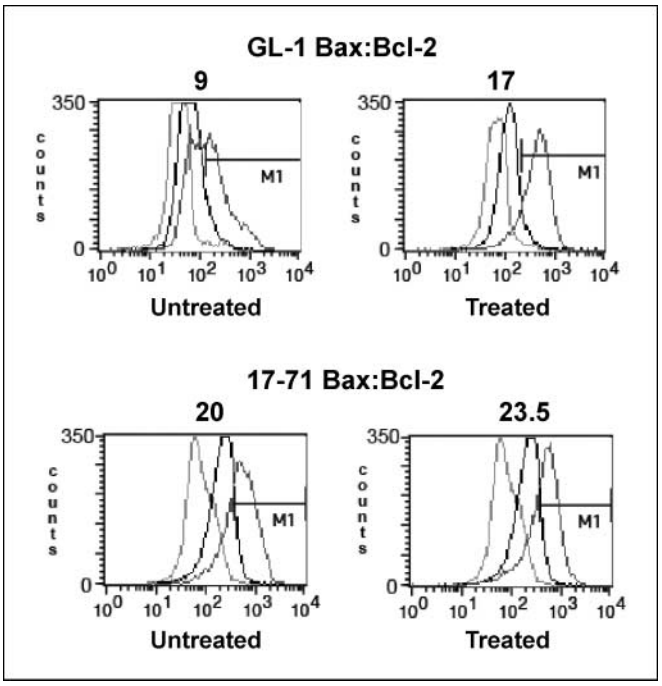

Fig. 5. GCP increases Bax/Bcl-2 ratios in (*A*) GL-1 and (*B*) 17-71 cells. Cells were exposed to the IC_{50} of GCP for 72 h. Adherent and floating cells were pooled, washed, permeabilized, and stained with anti-Bax and Bcl-2 antibodies as described and analyzed by flow cytometry. Histograms were generated and analyzed using CellQuest cell cycle software. In both *A* and *B*, left hand histograms represent cells treated with carrier only, whereas right hand histograms represent GCP- treated cells. In all histograms: brown tracings, unstained cells; black tracings, cells stained with the anti-Bcl-2 antibody; grey tracings, cells stained with anti-Bax antibody. Bax/Bcl-2 ratios were calculated as described.

***GCP modulates the Bax:Bcl-2 ratio in exposed canine cells* in vitro.** Genistein is documented to affect protein levels of the proapoptotic protein Bax and the antiapoptotic protein Bcl-2 *in vitro* (23). To determine if GCP-induced apoptosis was due, in part, to altered Bax:Bcl-2 ratios we used flow cytometry to assess the Bax:Bcl-2 ratio in GCP-treated canine cells. GL-1 cells and 17-71 cells were treated with the IC_{50} of GCP for 72 hours and stained with anti-Bax and anti-Bcl-2 antibodies. The calculated ratio of Bax:Bcl-2 protein expression of carrier-only and GCP-treated cells, normalized to carrier-only–treated cells, is shown in Fig. 5. In treated GL-1 cells, the Bax:Bcl-2 ratio was increased approximately 53%, whereas the ratio was increased approximately 17% in treated 17-71 cells.

Plasma concentrations of genistein in dogs fed increasing amounts of GCP were below the* in vitro *IC_{50}. Isoflavone glycosides, such a genistein, are not readily absorbed across enterocytes in most species, which limits their therapeutic use to i.v. administration. GCP, which is an aglycone isoflavone, is highly bioactive due to unimpeded intestinal absorption. Given its availability as a simple dietary supplement and the convenience of oral dosing in veterinary species, we performed a dose escalation pharmacokinetic study to determine if GCP fed to normal dogs led to plasma genistein levels that approached the *in vitro* IC_{50} of the compound and evaluate any toxicities associated with oral dosing. We fed 1.5, 3.5, and 5 grams (0.45%, 1.0%, and 1.45% GCP-supplemented diet, respectively) GCP to normal dogs with a dose level ranging from 0.05 mg/kg/day to 0.19 mg/kg/day. Plasma genistein levels were determined as described for 48 hours postfeeding whereas CBCs and serum biochemistries were analyzed before and 48 hours after feeding. In addition, the dogs were monitored clinically throughout the study and for 2 weeks thereafter for signs of GCP toxicity.

The results of the pharmacokinetics study are seen in Fig. 6 and Table 1. The plasma drug concentration-time data were fitted to a noncompartmental analysis. Although we were able to detect genistein within 1 hour of feeding, the plasma level rarely exceeded 0.26 μg/L, which is well below the *in vitro* IC_{50} documented in our previous *in vitro* experiments (10 μg/mL and 20 μg/mL for GL-1 and 17-71 cells, respectively). The mean genistein AUC for all three dogs was 0.1810 ± 0.0380, 0.3080 ± 0.1660, and 0.5480 ± 0.0177 hour × μg/mL when fed 1.5, 3.5, and 5.0 g GCP, respectively. The mean genistein half-life for all three dogs were 4.98 ± 1.23, 3.35 ± 0.77, and 5.1 ± 2.35 hours when fed 1.5, 3.5, and 5.0 g GCP, respectively. Additionally, the peak plasma level dropped significantly approximately 2 hours postfeeding and was undetectable at both 24 and 48 hours postfeeding (data not shown). Clinically, we did not observe any gastrointestinal (vomiting, diarrhea, anorexia), blood/bone marrow (CBC, serum chemistries), or constitutional signs (lethargy, fatigue, weight loss) toxicities throughout the entire course of this study.

Discussion

To our knowledge, this is the first reported study to document that GCP, a soy isoflavone extract fermented with *G. lucidum* and rich in aglycone isoflavones, has significant antiproliferative activities against canine lymphoid cell lines. Because approximately 85% of canine lymphoma patients develop B-cell lymphoma (38), we chose to examine two known canine B-cell lymphoid cell lines, GL-1 and 17-71. Additionally, because GCP is a commercially produced, orally bioavailable form of genistein, we developed a HPLC assay to detect canine plasma genistein levels and show that genistein in orally administered GCP is absorbed by enterocytes of normal dogs.

Our initial experiments revealed that both of the canine B-cell lymphoid cell lines, GL-1 and 17-71, were susceptible to the antiproliferative effects of genistein. Overall, the GL-1 cells were more sensitive to both genistein and GCP when compared with the 17-71 cells (IC_{50} of genistein and GCP at 72 hours for

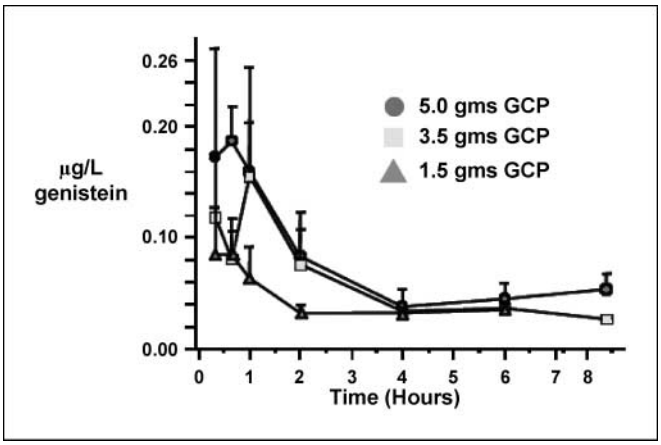

Fig. 6. Orally administered GCP leads to detectable genistein plasma levels in normal dogs ($n = 3$). Genistein plasma levels were obtained using HPLC and plotted against time (h) after 1.5, 3.5, and 5.0 g GCP were added to the dogs' food.

Table 1. The pharmacokinetic parameters Tmax, Cmax, AUC, and half-life were calculated for each dog when fed increasing amounts of GCP

Parameter	Dog 1			Dog 2			Dog 3		
	1.5 g	3.5 g	5.0 g	1.5 g	3.5 g	5.0 g	1.5 g	3.5 g	5.0 g
Tmax (h)	0.67	1.0	0.67	0.33	0.33	0.33	0.33	1.0	1.0
Cmax (μg/mL)	0.0547	0.0777	0.01412	0.106	0.076	0.252	0.055	0.2396	0.1554
AUC (h × μg/mL)	0.2096	0.2743	0.7195	0.1955	0.2125	0.372	0.1378	0.4377	0.5527
Half-life (h)	5.1997	2.9417	7.0874	3.6433	4.2442	2.5027	6.0987	2.8577	5.7273

GL-1 cells was 10 ug/mL compared with 17-71 cells with an IC_{50} of 20 ug/mL; Fig. 1). These findings are in agreement with human studies documenting cell line–specific differences in genistein responses (28, 39, 40). We also show that GCP was found to be a more potent inhibitor of cell growth in both cell lines when purified genistein was compared with equivalent concentrations of genistein in the GCP extract. The reasons for this are unclear, although other components of GCP, such as diadzein and glycitein, also have documented antiproliferative properties (41, 42). Because GCP, when prepared with a genistein concentration similar to the pure product, had a greater antiproliferative effect against the canine cell lines and is a commercially available, affordable oral form of the compound that can be easily added to food, we chose to use only GCP for all subsequent experiments.

In these studies, the dose-dependent antiproliferative effect of GCP against canine cells was due to apoptosis rather than cytostasis. Cell cycle analysis of treated cells documented an increased population of cells localizing to the sub-G_0 region of the histogram (Fig. 2) rather than an increase in the G_2-M region. Early apoptosis was verified using Annexin-V staining and caspase 9/3 activation, whereas evidence of late apoptosis was seen with significant DNA degradation into ~200 bp nucleosomes (laddering).

An important apoptotic pathway in lymphocytes is regulated by the balance of expression of proapoptotic and antiapoptotic proteins that are members of the Bcl-2 family (43). Bax, a proapoptotic protein, forms a heterodimer with Bcl-2 thereby diminishing the antiapoptotic activity of the Bcl-2 protein. Thus, it is the Bax:Bcl-2 ratio that determines the sensitivity of a cell to apoptosis where a low Bax/Bcl-2 ratio correlates with resistance to therapy (44). We chose to examine these proteins to determine if alterations in the Bax/Bcl-2 ratio are, in part, responsible for initiating the apoptotic cascade. In both treated cell lines the Bax:Bcl-2 ratio increased, although this effect was much more pronounced in the GL-1 cells (Fig. 5). These findings correlate with our cell cycle analysis and Annexin-V/7-AAD staining data (Figs. 2 and 3) suggesting that 17-71 cells undergo apoptotic cell death at a slower rate than GL-1 cells. Similar to human cell line data, we found that the increased Bax/Bcl-2 ratio in the GL-1 cells (and 17-71 cells to a lesser extent) was due to an increase in proapoptotic Bax protein expression rather than a decrease in antiapoptotic Bcl-2 protein expression (23, 40). Other cellular processes suggested to be involved in genistein-induced apoptosis include increased poly(ADP-ribose)polymerse cleavage (45), induced expression of p21 protein (46), and increased p53 phosphorylation (47). Further studies using these canine cells are needed to begin to mechanistically understand genistein-induced apoptosis.

Based on limited pharmacokinetic data showing that genistein metabolism and plasma levels vary widely between humans and rodents, we next sought to determine if genistein in orally administered GCP is absorbed across canine enterocytes by developing a sensitive HPLC assay. The amount of diet supplementation was based on previous murine studies (0.5-2.0% of diet; refs. 28, 29). The results of these studies (Fig. 6) are important on three fronts. Most importantly, even at low oral doses, we were able to measure detectable plasma levels of genistein, indicating that genistein, and possibly diadzein and glycitein, cross canine enterocytes and enter the systemic circulation. Second, our results show the extreme sensitivity of the HPLC assay, which can measure genistein levels as low as 0.01 μg/L. This will be important for future studies used to determine an appropriate dosing concentration and schedule. And finally, as seen in CD2F1 mice (48), it seems that gut-absorbed genistein is metabolized rapidly in dogs, with plasma levels dropping by one half within 2 hours of administration and disappearing completely within 24 hours. The short half-life found in these dogs fed GCP are similar to two previous canine studies examining the pharmacokinetics in beagle dogs force-fed pure genistein (48, 49). This implies, unlike human data suggesting genistein has an elimination half-life between 15 and 22 hours, that multiple daily doses may be needed in dogs to elicit appropriate antiproliferative responses.

In summary, these results support the notion that GCP has significant *in vitro* antiproliferative effects against canine B-cell lymphoid cell lines. Our initial pharmacokinetic data emphasize the importance of bioavailability and disposition kinetics studies when attempting to evaluate the *in vivo* effects of compounds in different species. Based on our data showing orally administered GCP leads to detectable genistein plasma levels, these results support the notion that genistein may be useful in both chemopreventive and treatment regimes for canine B-cell lymphoma. Based on this promising preliminary data, additional studies in our laboratory are ongoing in an effort to determine if canine B-cell lymphoma will be a relevant large-animal model for examining the *in vivo* efficacy of genistein for human diffuse large B-cell lymphoma treatment regimes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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