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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> The goal of our research was to determine if there is a developmental window for suppressing prostate cancer with the phytoestrogen, genistein, and its mechanism (s) of chemoprevention. Life-time (starting at birth) exposure to genistein was more effective in suppressing chemically-induced prostate cancer in rats than neonatallprepubertal or adult only exposures to genistein. Neonatallprepubertal genistein exposure did not alter prostate bud development. Dietary genistein during the neonatallprepubertal period as well as during adulthood resulted in decreased androgen receptor, but not estrogen receptors alpha and beta, in dorsolateral prostates of 70 day old rats, suggesting that early exposure to genistein can have a programming effect on androgen receptor expression. However, genistein at each period of postnatal development did not alter androgen receptor mRNA in the prostate, data that argues against genistein causing gene silencing via DNA methylation mechanism in the prostate of rats. On the other hand, genistein in the diet resulted in increased apoptosis in the prostate. Phospho-Akt and Bcl-2 protein levels were decreased and Bax was increased in prostates of rats fed genistein in ttle diet, protein regulation consistent with increased apoptosis, a cellular mechanism that can suppress prostate cancer development.					
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## Introduction

The disease of cancer is usually attacked at time of diagnosis, and even chemoprevention is not usually considered until adulthood. Our hypothesis is that windows of development hold the key for chemoprevention of prostate cancer. We have previously demonstrated that exposure to physiological concentrations of genistein starting at 5 weeks suppressed the development of chemically-induced prostate cancer and genistein is bioavailable to the prostate in rats. The purpose of our proposed research is to determine if there is a developmental window for this chemoprevention and the mechanism(s) of chemoprevention. The importance of this lies in the need to know, prior to initiation of human trials, if we need to expose infants and/or adults to get maximum chemoprevention.

## Body

**Aim 1.** To determine if a specific window of development (neonatal/prepubertal only, adult only or life-time) is responsible for genistein chemoprevention of prostate cancer. This was carried out in the following groups of rats.

Group A) no genistein in the diet as positive controls.

Group B) genistein in the diet from birth until 35 days of age only.

Group C) genistein in the diet from 90 to 330 days.

Group D) genistein via the diet from birth throughout life to demonstrate that postnatal lifetime exposure only protects against prostate cancer.

Table 1. Prostate Cancer Incidence in Lobund-Wistar Rats\* Fed Genistein in the Diet

Treatment	#/Group	Percent of Rats with Pathological Score:					
		1	2	3	4	5	6
A) Controls	23	22	4	17	4	9	43
B) Genistein (1-35 Days)	27	37	4	15	7	7	30
C) Genistein (3-11 Months)	28	32	25	14	0	0	29 <sup>a</sup>
D) Genistein (1-11 Months)	30	43	23	0	3	10	20 <sup>a,b</sup>

\*Lobund-Wistar male rats were gavaged with 33 mg flutamide/kg BW on days 50-66 postpartum to cause chemical castration. On days 67, 68 and 69 they were injected with 25 mg testosterone/kg BW to stimulate mitosis. On day 70, they were injected with 42 mg NMU/kg BW into the dorsal prostate to initiate cancer. One week after NMU administration, silastic implants of 25 mg testosterone were administered (and replaced every 12 weeks) to simulate mitosis and promote tumor growth. Rats were necropsied when 11 months old or when moribund. Key to Pathology report: 1 – Normal tissue; 2 – Low-grade PIN; 3 – High-grade PIN; 4 – Well-differentiated lesion; 5 – Moderately differentiated lesion; 6 – Poorly differentiated lesion. <sup>a</sup> < 0.05 compared to controls based on the exact Cochran-Armitage trend test. <sup>b</sup> < 0.05 compared to Controls only using the chi-square test.

From a 4 x 6 contingency table analysis based on the exact likelihood ratio chi-square test, there was significant association between treatments (Controls, Genistein for 1-35 Days, Genistein for 3-11 Months, and Genistein for 1-11 Months) and tumor grade (1-6), p-value = 0.022. However, when comparing each of the treatment groups to the controls, only Genistein for 1-11 months was significantly different than controls (p-value = 0.023). Note that while 43.5% of the Control animals had grade-6 tumors, only 20% of the Genistein for 1-11 months and while only 21.7% of the Control animals had no tumors (grade-1), 43.3% of the Genistein for 0-11 months had no

tumors (grade-1). Genistein for 1-35 days and Genistein for 3-11 months were not significantly different than controls (p-values = 0.887 and 0.072, respectively). In addition, there were no significant differences between Genistein for 1-11 months and Genistein for 1-35 days (p-value=0.075). No other comparisons were significant.

Based on the exact Cochran-Armitage trend test, the Genistein 1-11 month and the Genistein 3-11 month groups had statistically significant downward trends in tumor grade as compared to controls (p-value =0.016, 0.041, respectively). There was no significant trend for Genistein 1-35 days as compared to controls (p-value=0.134). There were no significant differences in trend between any of the Genistein treatment groups.

**Aim 2.** To investigate prostate gland morphology in the dorsal- and lateral-lobes of the prostates of 21 and 35 day old rats exposed ± genistein in the diet, starting at birth.

Prostate Bud Perimeter (mm) in 21 Day Old Male Rats*			
	<u>DP</u>	<u>LP1</u>	<u>LP2</u>
Control	0.65 ± 0.01	0.67 ± 0.02	0.61 ± 0.02
Genistein	0.66 ± 0.02	0.61 ± 0.02	0.60 ± 0.02

Prostate Bud Perimeter (mm) in 35 Day Old Male Rats*			
	<u>DP</u>	<u>LP1</u>	<u>LP2</u>
Control	1.13 ± 0.03	1.15 ± 0.04	1.05 ± 0.03
Genistein	1.14 ± 0.03	1.15 ± 0.03	0.97 ± 0.02

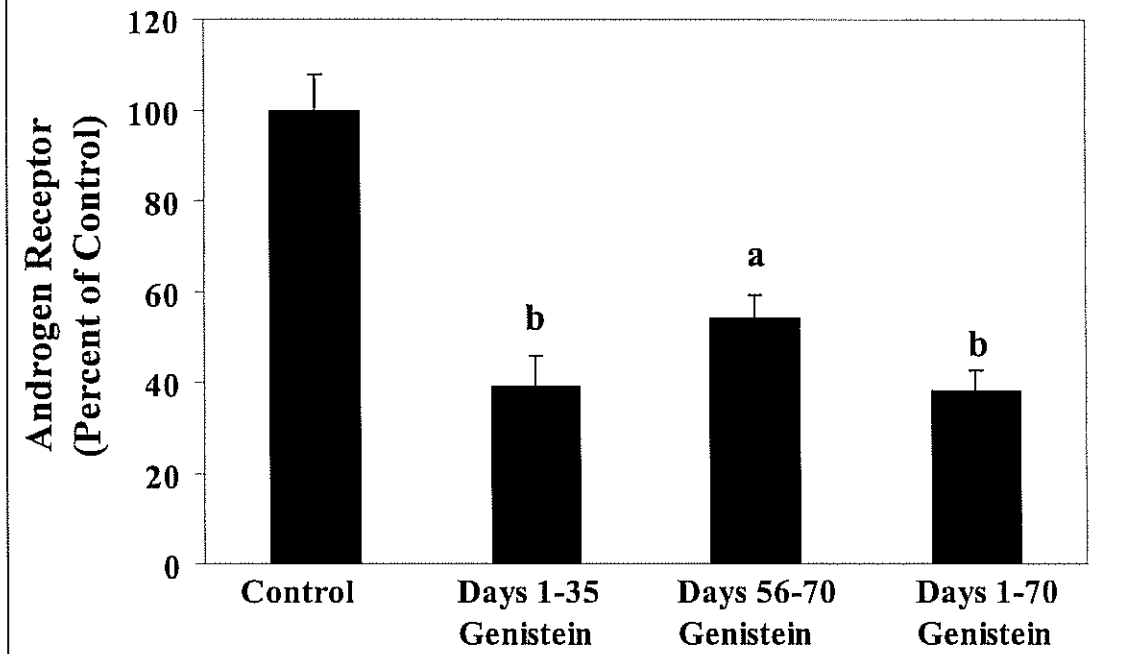
\*These 21 and 35 day old rats were exposed to 250 mg genistein/kg AIN-76A diet, starting at birth. DP: dorsal prostate; LP1: lateral prostate lobe 1; LP2: lateral prostate lobe 2. No statistical significance was detected for prostate bud perimeter from genistein compared to control treated rats using ANOVA.

These data demonstrate that neonatal/prepubertal genistein in the diet did not alter prostate gland development and is not the cellular mechanism of chemoprevention.

**Aim 3.** To investigate the potential of genistein to regulate sex steroid receptor expression as mechanism of prostate cancer prevention. This was carried out in 70 day old rats, time of carcinogen (NMU) exposure.

Dietary genistein resulted in decreased androgen receptor (Fig. 1), but not estrogen receptors alpha and beta (data not shown), in dorsolateral prostates of 70 day old rats. The novel finding is that neonatal/prepubertal short term genistein treatment (days 1-35) was able to down-regulate the androgen receptor as well as did adult only (days 56-70) and life-time (days 1-70) genistein treatments. Since short term early exposure to genistein is as effective as short term late and lifetime exposure to genistein, this suggest that early exposure to genistein can have a programming or imprinting effect on the androgen receptor. This is consistent with neonatal/prepubertal genistein treatment causing permanent effect on gene and protein expression via DNA methylation or on other effects that indirectly regulate androgen receptor expression.

**Fig. 1. Androgen Receptor Expression in Dorsolateral Prostates of Rats Fed Genistein in the Diet**



Androgen receptor protein was determined via Western blot analysis. Each group contained 8 rats. <sup>a</sup>P < 0.01 and <sup>b</sup>P < 0.001 compared to controls.

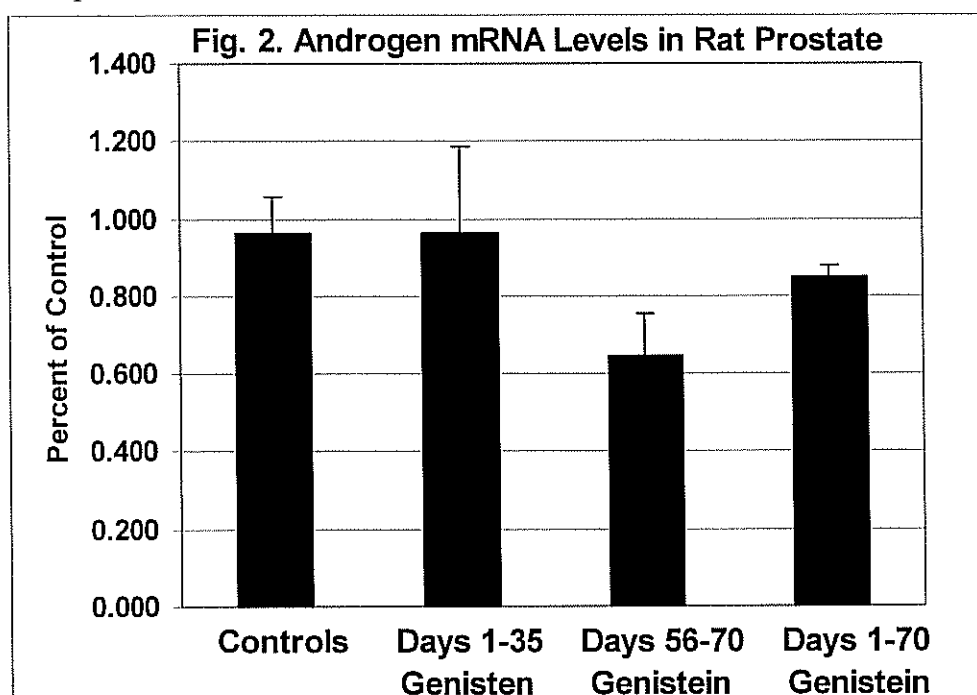
**Aim 4.** To investigate DNA methylation of androgen receptor as imprinting mechanism of action.

Based on the data of Aim 3, it is plausible that androgen receptor, but not estrogen receptors alpha and beta, could be regulated via DNA methylation mechanism. Hence, we attempted to measure DNA methylation via methylation specific PCR (MSP). However, rat androgen receptor MSP primers have not been previously designed. While we designed two sets of methylated and unmethylated primers based on the rat androgen receptor promoter region sequence, we were not able to demonstrate DNA methylation (data not shown).

As a final attempt to determine if gene silencing occurred, we used real-time PCR to measure gene expression levels of the rat androgen receptor. The Applied Biosystems 7500 Real-Time (RT) PCR System was utilized to measure gene expression levels of the rat androgen receptor (Applied Biosystems, Foster City, CA). Briefly, total RNA was extracted from prostate tissue *via* Qiagen RNeasy Mini kit according to the manufacturer's protocol (Qiagen, Valencia, CA). We used One-Step TaqMan RT-PCR chemistry which relies on the 5'-nuclease activity of DNA polymerase and the Förster Resonance Energy Transfer (FRET) of a reporter and quencher dye labeled with FAM-MGB to generate the fluorescence signal. Universal master mix, multiscribe reverse transcriptase and RNase inhibitors, the primer and probe mix, and template RNA were loaded onto a 96 well plate. Primer and probe combinations for the rat androgen receptor (Assay ID: Rn00560747\_m1) were purchased from Applied Biosystem's Assay on Demand (Applied Biosystems). Rat S9 (Assay ID: Rn01530912\_g1), a mitochondrial ribosomal protein, was used as an endogenous control to normalize mRNA concentrations. Rat S9 is an ideal endogenous

control since it has a constant RNA transcription level under different experimental settings and is expressed consistently across different tissues. Non-template controls (devoid of mRNA) were implemented to monitor pipette carryover. Each sample was run in triplicate for the target gene and the endogenous control. A minimum of eight samples per group was analyzed. The relative quantification (RQ) assay, a real-time quantitative PCR application, did not reveal gene expression changes in the treated samples relative to calibrator (control) samples (data not shown).

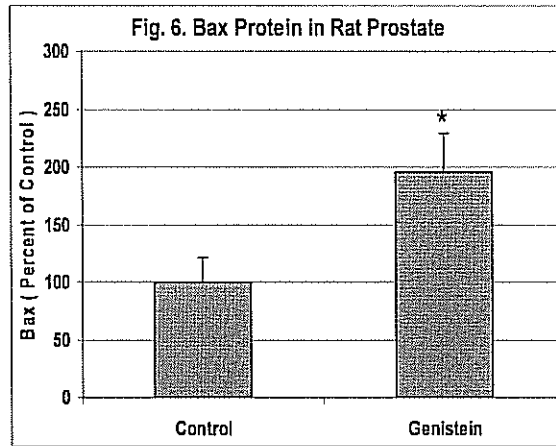
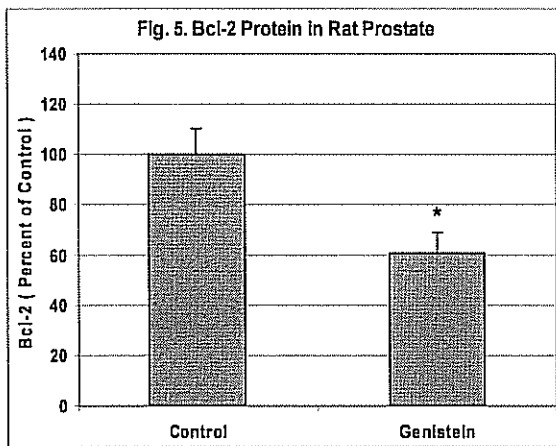
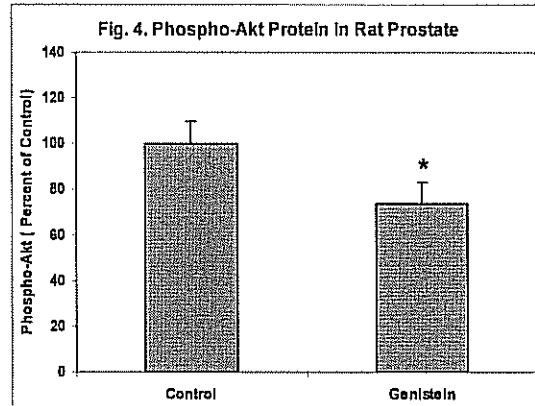
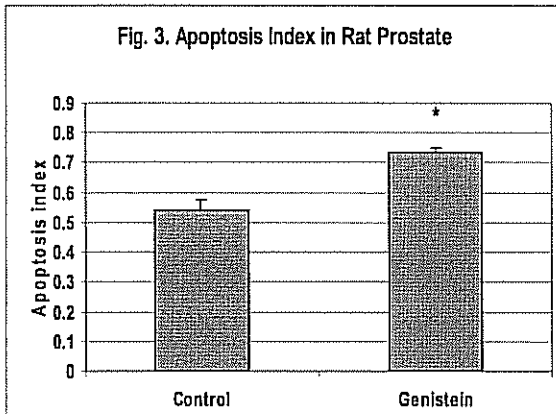
As seen in Figure 2, androgen receptor mRNA levels from prostates of rats exposed 1) neonatally/prepubertally only (days 1-35), 2) for two weeks postpubertally (days 56-70) or 3) from days 1-70 were not significantly different from controls (exposed to AIN-76A diet only). Hence, we conclude that genistein did not effect gene silencing via DNA methylation mechanism in the prostate of rats.



Androgen receptor mRNA was determined *via* real-time PCR. Each group contained 6 rats. Using ANOVA, no significantly difference was found compared to controls.

Additional work. We have also investigated the potential of genistein in the diet from days 1-70 to alter apoptosis. Figure 3 demonstrates that there is significantly increased cell death in prostates of rats fed genistein. One protein that plays a role in cell survival is AKT (Protein Kinase B). The phosphorylated product subsequently recruits Akt family members to the inner leaflet of the plasma membrane and stimulates their protein kinase activity. Measurement of phospho-Akt protein by Western blot analysis revealed significantly reduced levels in prostates of rats fed genistein supporting the fact that apoptosis was increased (Fig. 4). Two other proteins that factor in cell survival are Bcl-2 and Bax. The two proteins have highly similar amino acid sequences but are functionally opposed: Bcl-2 acts to inhibit apoptosis, whereas Bax counteracts this effect. The antagonism appears to depend upon dimerization between Bcl-2 and Bax, but its mechanism is otherwise unknown. Overexpressed Bax accelerates apoptotic death. The ratio of bcl-2 to Bax determines survival or death after an apoptotic stimulus. In rats fed genistein we found that Bcl-2 was down regulated and Bax was up-regulated (Figures 5 and 6). This is

consistent with genistein increasing apoptosis and suppressing prostate cancer development in the rat model.



The TdT-FragEL™ DNA Fragmentation Detection Kit (Calbiochem, San Diego, CA) was used to measure apoptosis following the manufacturer's instructions. Phospho-Akt, Bcl-2 and Bax proteins were determined via Western blot analysis. Each group contained 8 rats. \*P < 0.05 compared to controls.

### Key Research Accomplishments

- There was significant association between treatments (Controls, Genistein for 1-35 Days, Genistein for 3-11 Months, and Genistein for 1-11 Months) and tumor grade (1-6), p-value = 0.022.
- Based on the exact Cochran-Armitage trend test, the 1-11 month Genistein and the 3-11 month groups Genistein had statistically significant downward trends in tumor grade as compared to controls (p-value = 0.016, 0.041, respectively).
- Compared to Controls, only Genistein for 1-11 months was significantly different in tumor grade (p-value = 0.023).



- Neonatal/prepubertal genistein in the diet did not alter prostate bud perimeter in 21 and 35 day old rats. This demonstrates that genistein is not capable of altering rat prostate morphology.
- Dietary genistein during the neonatal/prepubertal period as well as during adulthood resulted in decreased androgen receptor, but not estrogen receptors alpha and beta, in dorsolateral prostates of 70 day old rats, suggesting that early exposure to genistein can have a programming or imprinting effect on androgen receptor expression.
- Genistein in the diet at each period of postnatal development did not alter androgen receptor mRNA in the prostate, data that argues against genistein causing gene silencing via DNA methylation mechanism in the prostate of rats.
- Genistein in the diet resulted in increased apoptosis in the prostate.
- Phospho-Akt and Bcl-2 protein levels were decreased and Bax was increased in prostates of rats fed genistein in the diet, protein regulation consistent with increased apoptosis.

### **Reportable Outcomes**

Molecular and Cellular Pathology Seminar: Dietary Polyphenols Protect Against Mammary and Prostate Cancers. University of Alabama at Birmingham Department of Pathology. September, 2005.

University of Gottingen, Germany Seminar: Dietary Polyphenols Protect Against Mammary and Prostate Cancers. February, 2006.

Humboldt University, Berlin, Germany Seminar: Dietary Polyphenols Protect Against Mammary and Prostate Cancers. February, 2006.

Technische Universitat, Dresden, Germany Seminar: Dietary Polyphenols Protect Against Mammary and Prostate Cancers. February, 2006.

University of Alabama at Birmingham Hematology and Oncology Seminar: Dietary Polyphenols Protect Against Mammary and Prostate Cancers. March, 2006.

Wang, J., Eltoun, I.A. and Lamartiniere, C.A. Genistein suppresses prostate cancer in chemically-induced Lobund-Wistar rat model. In preparation for International Journal of Cancer.

### **Conclusion**

Genistein in the diet suppressed chemically-induced prostate cancer in rats. Life-time (starting at birth) exposure to genistein is more effective in conferring protection against prostate cancer than neonatal/prepubertal or adult only exposures to genistein, suggesting that developmental and/or programming effects plus maintenance regulation plays a role in protecting against prostate cancer. Neonatal/prepubertal genistein exposure does not alter prostate bud development. Dietary genistein during the neonatal/prepubertal period as well as during adulthood resulted in decreased androgen receptor, but not estrogen receptors alpha and beta, in

dorsolateral prostates of 70 day old rats, suggesting that early exposure to genistein can have a programming or imprinting effect on androgen receptor expression. However, genistein at each period of postnatal development did not alter androgen receptor mRNA in the prostate, data that argues against genistein causing gene silencing via DNA methylation mechanism in the prostate of rats. On the other hand, genistein in the diet resulted in increased apoptosis in the prostate. Phospho-Akt and Bcl-2 protein levels were decreased and Bax was increased in prostates of rats fed genistein in the diet, protein regulation consistent with increased apoptosis, a cellular mechanism that can suppress prostate cancer development.

### **References**

None

### **Appendices**

List of personnel receiving pay from the research effort

## **Appendix**

### **List of personnel receiving pay from the research effort**

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Dr. Jun Wang, M.D, Res. Instructor

Natalie Durr, BS, Technician

Glen Puckett, BS, Technician

Hongming Liu, BS, Technician