

# Preventive effects of Polyphenon E on urinary bladder and mammary cancers in rats and correlations with serum and urine levels of tea polyphenols

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## Abstract

Polyphenon E, a standardized mixture of green tea polyphenols, was examined for its chemopreventive efficacy against chemically induced urinary bladder and mammary cancers. In the present study, Polyphenon E was administered after the last dose of 4-hydroxybutyl(butyl)nitrosamine, or roughly 30% of the way into the experiment. Polyphenon E (100 or 250 mg/kg body weight/d) caused a dose-dependent decrease in palpable urinary bladder tumors [low dose, 14 of 34; high dose, 6 of 35; controls, 20 of 34 ( $P < 0.01$ )]. In the mammary cancer model, Polyphenon E [333 or 1,000 mg/kg body weight (BW)/d] was administered beginning 5 days after a single dose of methylnitrosourea. In contrast to its significant efficacy in bladder tumor prevention, Polyphenon E had a minimal effect in the prevention of mammary cancers. Levels of polyphenols were determined in the urine and serum of rats. Relatively high levels of various polyphenols (and metabolites) were found in the urine. However, virtually no epigallocatechin-3-gallate was observed in the urine because of low systemic bioavailability; although it represents almost 65% of the polyphenols in Polyphenon E. Levels of polyphenols in serum were  $50\times$  to  $1,000\times$  less than were observed in urine. The bioavailability of

these tea polyphenols to different organ sites may contribute to the differing preventive efficacy of Polyphenon E against urinary bladder and mammary cancers. [Mol Cancer Ther 2007;6(7):2022–8]

## Introduction

Two chemically induced rat cancer models were used to determine the efficacy of Polyphenon E as a chemopreventive agent—the methylnitrosourea (MNU)–induced mammary model and the 4-hydroxybutyl(butyl)nitrosamine (OH-BBN)–induced urinary bladder model. Induction of cancers by the organ-specific carcinogen OH-BBN is the most commonly used method for bladder tumor induction in rodents (1, 2). The tumors have a mixed histology with most appearing to be transitional cell carcinomas. This model has been used extensively to evaluate agents that might prevent urinary bladder cancer (1–4). A wide variety of nonsteroidal anti-inflammatory drugs, such as piroxicam, indomethacin, and the selective cyclooxygenase-2 inhibitor celecoxib, have proven to be profoundly effective in inhibiting the formation of OH-BBN–induced cancers. In a previous study from our laboratory, intervention with celecoxib after the final treatment with OH-BBN was shown to be highly effective (3). Based on this ability of celecoxib to inhibit bladder tumors even when administered late during the cancer process, we have used this delayed treatment protocol in studies with many other potential preventive agents.

Chemically induced models of mammary carcinogenesis were initially developed by Huggins et al. (5). Female Sprague-Dawley rats treated with MNU or dimethylbenzanthracene develop multiple hormonally responsive mammary cancers starting within 5 weeks after the administration of the carcinogen at 50 days of age (5, 6). The tumors seem to be histologically (and by gene expression) similar to well-differentiated estrogen receptor–positive mammary cancers in women (7). As might be expected, agents or conditions that alter the hormonal axis (e.g., selective estrogen receptor modulators, aromatase inhibitors, and pregnancy) are strong chemoprevention modifiers in this model. In addition, it has been shown that this model responds to various agents that may be independent of the hormonal axis, including a variety of retinoid X receptor agonists, epidermal growth factor receptor inhibitors, and farnesyltransferase inhibitors (8, 9).

Tea polyphenols have shown inhibitory activity against a variety of cancers in animal models (10–12). Polyphenon E has not been previously examined for its ability to alter urinary bladder carcinogenesis. However, green tea leaves (of which polyphenols represent roughly 15% of dry weight) have shown efficacy against bladder cancer

Received 1/26/07; revised 5/1/07; accepted 5/25/07.

**Grant support:** National Cancer Institute contract numbers NO1-CN-43308 and NO1-CN-25115 and National Cancer Institute grants RO1-CA101240, RO1-CA096131, and PO1-CA88961.

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doi:10.1158/1535-7163.MCT-07-0058

induction (13, 14). Tea catechins administered in the diet of rats dosed with dimethylbenzanthracene reduced the volume of mammary tumors (15). Kavanagh et al. (16) reported that green tea increased latency to first mammary tumor, but did not affect tumor number in female Sprague-Dawley rats receiving dimethylbenzanthracene. In contrast, epigallocatechin-3-gallate (EGCG), the major catechin found in green tea, failed to suppress mammary carcinogenesis induced with dimethylbenzanthracene (17). The effects of Polyphenon E in inhibiting OH-BBN-induced bladder cancer and MNU-induced mammary cancer were determined in this study. In addition, we determined urinary and serum levels of various tea polyphenols and certain of their metabolites.

## Materials and Methods

### Animals and Chemicals

Female Fisher-344 and Sprague-Dawley rats were obtained from Harlan Sprague-Dawley, Inc., at 28 days of age and were housed in polycarbonate cages (five per cage). The animals were kept in a room lighted 12 h each day and maintained at 22°C. Teklad (4%) diet (Harlan Teklad, Madison, WI) and tap water were provided *ad libitum*. Polyphenon E was supplied by the National Cancer Institute/Division of Cancer Prevention repository. OH-BBN was purchased from TCI America and MNU was purchased from the Division of Cancer Prevention and Control chemical repository (Midwest Research Institute). Dimethylbenzanthracene was obtained from Sigma Chemical Company. Polyphenon E is a standardized green tea polyphenols mixture prepared by the Mitsui Norin Co., and contains 64.3% EGCG, 3.1% (–)-epigallocatechin (EGC), 9.1% (–)-epicatechin (EC), 8.1% (–)-epicatechin-3-gallate, and other polyphenols. The chemical structures of the primary polyphenols in Polyphenon E are presented in Fig. 1.

### Urinary Bladder Cancer Model

OH-BBN (150 mg/gavage) was administered twice weekly for 8 weeks beginning when the female Fischer-344 rats were 56 days of age. OH-BBN was administered (1.0 mL/gavage) in ethanol/water (25:75). Polyphenon E was initially administered when the rats were 126 days of age or 1 week after the final dose of OH-BBN. Polyphenon E (250 or 100 mg/kg BW/d) was administered in saline by gavage (0.5 mL/gavage) 7 d/wk. The rats were observed daily, weighed weekly, and palpated for urinary bladder lesions twice weekly. Rats were sacrificed when they developed a large palpable bladder lesion or were observed to have bloody urine. At necropsy, urinary bladders were inflated with 10% buffered formalin. After fixation, the bladder was observed under a high intensity light for gross lesions. Each lesion was dissected and processed for pathologic classification. The Kaplan-Meier test was used to analyze survival data. Urinary bladder cancer incidence was analyzed by the Fisher's exact test.

### Mammary Cancer Model

At 50 days of age, female Sprague-Dawley rats received one i.v. injection of MNU (75 mg/kg BW) via the jugular

vein. MNU was dissolved in saline (adjusted to pH 5.0 with 3% acetic acid) immediately before administration to the rats. Polyphenon E was given by gavage beginning 5 days after treatment with the carcinogen. Polyphenon E was administered seven times per week at dose levels of 1,000 and 333 mg/kg BW/d. A previous dose selection study lasting 6 weeks had indicated that these doses were not toxic in this strain of rats. Vaginal smears were taken for a 2-week period at 2 months after the initial administration of Polyphenon E to determine any effect on estrus cycles. The animals were weighed once per week and palpated for mammary tumors twice per week. The location of any mammary tumor was recorded such that, after histologic diagnosis, the time of appearance of the cancers could be plotted. All mammary tumors and gross lesions were processed for histologic classification. Mammary cancers were classified as adenocarcinomas, whereas benign mammary tumors included fibromas, adenomas, and fibroadenomas. The Armitage test was used to compare the average number of cancers per rat; the log-rank test was used to compare tumor incidence rates; and the Student's *t* test was used to compare tumor weights.

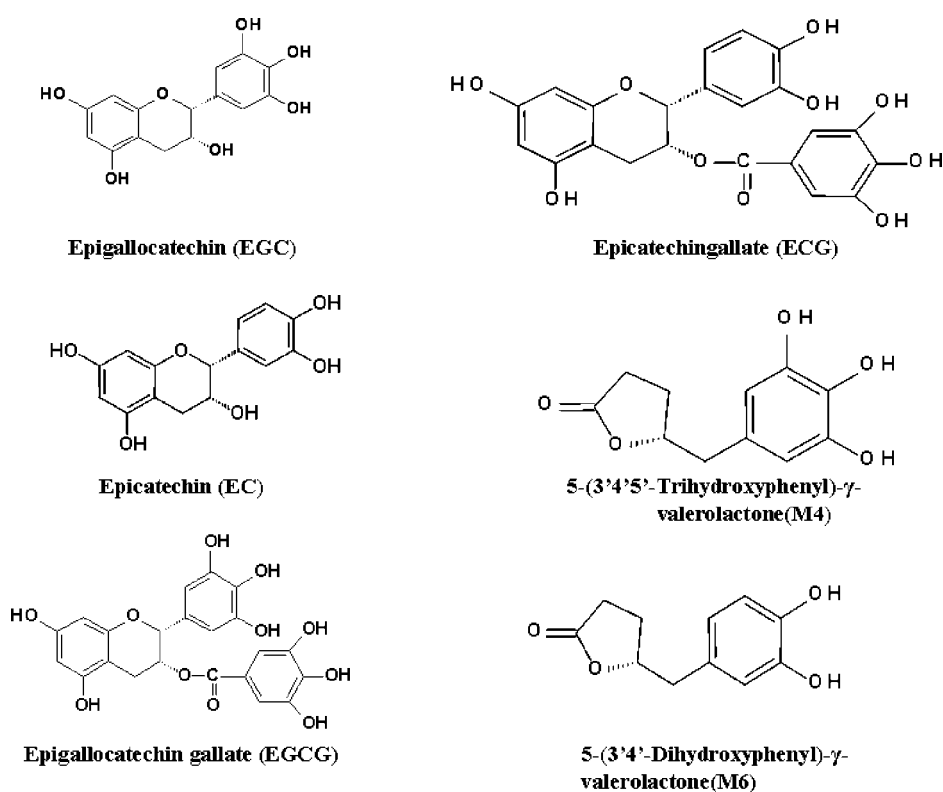
### Analysis of Urine or Serum for Polyphenols and their Metabolites

Female Sprague-Dawley rats were treated with Polyphenon E (250 mg/kg BW/d) by gavage once daily for 14 days. On the last day of the study (at 8 and 16 h after the last dose of Polyphenon E), urine was collected using metabolism cages; urine fell into tubes surrounded by dry ice. At sacrifice, blood was collected and centrifuged, and the serum was frozen at –85°C until analyzed. Urinary levels of polyphenols were analyzed using our previous procedures (18, 19). In brief, the thawed urine samples (50 µL) were hydrolyzed with a mixture of β-D-glucuronidase (250 units) and sulfatase (1 unit) to convert the glucuronidated and sulfated metabolites to the aglycons. The reaction mixture was extracted twice with ethyl acetate, dried, redissolved in 10% acetonitrile aqueous solution, and then analyzed in an high-performance liquid chromatography system consisting of a Waters 717 plus refrigerated auto sampler, two ESA Model 582 pumps, and an ESA 5500 Coulochem electrode array system. The potential of the Coulochem electrode array system was set at –100, 100, 300, and 500 mV. A Supelcosil C-18 reversed-phase column 5 µm (150 mm × 4.6 mm ID) was used. For binary gradient elution, mobile phase A consisted of 30 mmol/L monobasic sodium phosphate acid. Mobile phase B consisted of 15 mmol/L monobasic sodium phosphate containing 58.5% acetonitrile and 12.5% tetrahydrofuran (pH 3.45). The total flow rate was maintained at 1.0 mL/min throughout the run.

## Results

### Effect of Polyphenon E on Urinary Bladder Cancers

As shown in Table 1, 20 of 34 rats given only OH-BBN had large (>5 mm diameter) palpable urinary bladder cancers. Of these, only seven developed palpable tumors before the scheduled sacrifice. Large bladder tumors were



**Figure 1.** Chemical structure of major tea polyphenols.

easily identified at the time of final sacrifice of the animals. The efficacy of the chemopreventive agents was, therefore, based on these large cancers. By using a late sacrifice when roughly 50% of OH-BBN control rats had developed large palpable tumors, virtually all rats had early lesions, for example, hyperplasias, papillomas, or microscopic cancers.

Polyphenon E was administered by gavage at two different doses (Table 1) beginning 1 week after the last dose of OH-BBN. Rats administered the low dose of Polyphenon E developed large cancers in 14 of 25 rats (40%), which was lower, but not statistically different, than control rats (20 of 34). However, in rats treated with the high dose of Polyphenon E, only 6 of 34 rats developed large cancers—a difference that was statistically significant

( $P < 0.01$ ). Even at the highest dose of Polyphenon E, there were no effects on body weight gain of the rats or other clinical signs of toxicity. Thus, a strong dose-dependent preventive effect was observed. Rats in a celecoxib control treatment group treated simultaneously with this Polyphenon E study developed large cancers in only 2 of 35 rats (data not shown).

#### Effect of Polyphenon E in Mammary Cancer Model

An initial study of Polyphenon E was done in our laboratories at dose levels of 400 and 133 mg/kg BW/d in the MNU-induced mammary cancer model. Because limited or no effect on prevention was observed (data not shown), the present study was done at doses of 1,000 and 333 mg/kg BW/d. The female Sprague-Dawley rats

**Table 1.** Effect of Polyphenon E on OH-BBN – induced urinary bladder cancers

Group	Carcinogen*	Treatment <sup>†</sup>	Large cancers <sup>‡</sup>	Percent of rats with microscopic lesions (%) <sup>§</sup>
1	OH-BBN	Polyphenon E, 250 mg/kg BW/d	6/34 (18%) <sup>  </sup>	100
2	OH-BBN	Polyphenon E, 100 mg/kg BW/d	14/35 (40%)	100
3	OH-BBN	None	20/34 (59%)	100

\*OH-BBN was administered to rats twice per week for 8 wks beginning when the animals were 56 d of age ( $n = 30$  rats per group).

<sup>†</sup>Chemopreventive agents were administered beginning 1 wk after the final OH-BBN treatment.

<sup>‡</sup>The study was terminated 7 mo after the final OH-BBN treatment. Large cancers were those that were >5 mm in diameter and filled >50% of the urinary bladder lumen.

<sup>§</sup>Percentage of rats with microscopic lesions (hyperplasia, papilloma, cancer).

<sup>||</sup>Significantly different from group 4 ( $P < 0.05$ ).

**Table 2. Effect of Polyphenon E in the prevention of MNU-induced mammary cancers**

Group	Carcinogen*	Treatment <sup>†</sup>	Mammary adenocarcinomas <sup>‡</sup>		
			Percent incidence	Number/rat	Average of weight cancers (g)
1	MNU	Polyphenon E, 1,000 mg/kg BW/d	87	4.2 (14%↓) <sup>§</sup>	6.2 (30%↓)
2	MNU	Polyphenon E, 333 mg/kg BW/d	93	4.2 (14%↓)	7.0 (21%↓)
3	MNU	No treatment	100	4.9	8.9

\*Female Sprague-Dawley rats received MNU at 50 d of age ( $n = 15$  rats per group).

<sup>†</sup>Chemopreventive agents were administered beginning when the rats were 55 d of age.

<sup>‡</sup>Data on mammary cancers were obtained at necropsy of the rats (126 d after MNU).

<sup>§</sup>Numbers in parentheses are percentage decreases from control group (group 3).

receiving only the carcinogen developed a mammary cancer multiplicity of 4.9 cancers per rat with an average cancer weight of 8.9 g (Table 2). The effect of Polyphenon E on the latency period of the mammary tumors is shown in Fig. 2. The high and low doses of Polyphenon E decreased the number of mammary cancers by 14% and reduced the weight of the cancer by 30% and 21%, respectively. These reductions were not statistically different. Even at the highest dose, there were no effects on body weight gain of the rats or other clinical signs of toxicity. Furthermore, Polyphenon E did not alter estrus cycle length at either dose level.

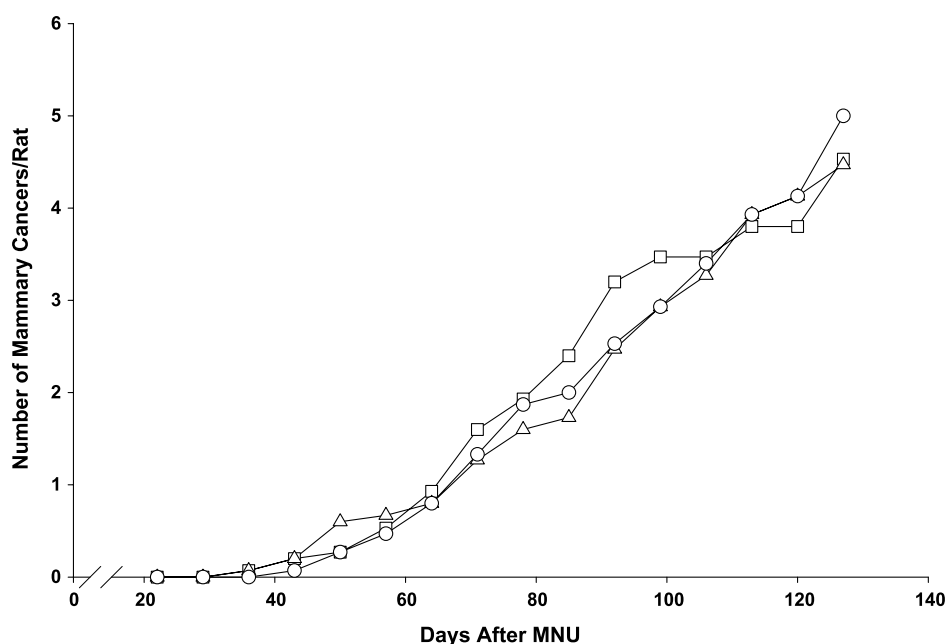
#### Polyphenol Levels in Urine and Serum of Treated Rats

The levels of various catechins or their metabolites were determined in both the serum and urine of female Fischer-344 rats either 8 or 16 h after the last gavage of Polyphenon E (250 mg/kg BW/d). In the urine, levels of the catechins EGC and EC were found to be quite high ( $\sim 20 \mu\text{g/mL}$ ) at both time points after Polyphenon E administration (Table 3). Virtually no EGCG was observed although it is the major polyphenol in Polyphenon E (64%). Two ring

fission metabolites of catechins, M4 and M6, were also observed in the urine samples (Fig. 1). Levels in serum were lower, with maximal levels of EGC and EC being 131 and 741 ng/mL, respectively, at 8 h (Table 4). These levels decreased greatly between 8 and 16 h after treatment, in contrast to the minimal difference in the urine samples collected at 8 and 16 h after Polyphenon E administration. EGCG was observed in the serum of treated rats; however, the levels were quite low ( $<30 \text{ ng/mL}$ ).

#### Discussion

The MNU-induced mammary cancer model and the OH-BBN-induced urinary bladder cancer model have been used in our laboratories to examine the preventive efficacy of a wide variety of agents. As mentioned in the Introduction, the bladder tumors in the OH-BBN model are composed primarily of a mixture of transitional cell and squamous cell cancers (1–3). These cancers have undergone molecular characterization; our laboratories, for example, have recently shown that fragile histidine triad (FHIT) expression is decreased and that there is increased



**Figure 2.** Effect of Polyphenon E on mammary cancer multiplicity in female Sprague-Dawley rats administered MNU. The groups were as follows: □, Polyphenon E (1,000 mg/kg BW/d); △, Polyphenon E (333 mg/kg BW/d); ○, controls.

**Table 3. Urinary levels of polyphenols and their metabolites**

Groups	(-)-Epigallocatechin	(-)-Epicatechin	M4*	M6*
Vehicle only	0.21 ± 0.05 <sup>†</sup> (0.7 μmol/L)	1.5 ± 1.4 (6.1 μmol/L)	0.14 ± 0.22 (0.1 μmol/L)	4.0 ± 2.5 (2.6 μmol/L)
Polyphenon E, 250 mg/kg BW/d (8 h)	21.8 ± 2.7 (70 μmol/L)	26.8 ± 1.0 (94 μmol/L)	3.68 ± 2.0 (2.2 μmol/L)	32.6 ± 9.3 (20 μmol/L)
Polyphenon E, 250 mg/kg BW/d (16 h)	18.8 ± 3.7 (62 μmol/L)	26.5 ± 0.4 (93 μmol/L)	4.5 ± 1.8 (2.6 μmol/L)	41.5 ± 13 (27 μmol/L)

\*M4, 5-(3',4',5'-trihydroxyphenyl)-γ-valerolactone; M6, 5-(3',4'-dihydroxyphenyl)-γ-valerolactone.

<sup>†</sup>Values are μg/mL (and μmol/L) and are the mean ± SD obtained at termination of the study from rats not receiving the carcinogen.

expression of survivin (20). These proteins are similarly altered in human bladder cancers. More recently, using RNA and protein techniques, we have found a wide variety of relevant genes to be altered in their expression, including *Ki67*, *Cyclin D1*, *GST Pi*, *Annexin A1*, and various calcium binding proteins (S100 A 4,8,9; ref. 21).

We have used this model to study prevention of urinary bladder cancer by a wide variety of agents. In this study of Polyphenon E (Table 1), 20 of 34 rats treated with OH-BBN only developed large palpable bladder lesions. The palpable tumors that developed during the study were normally preceded by the detection of bloody urine. These tumors were quite large (>5 mm in diameter) and filled >35% of the lumen. All these lesions were histologically classified as transitional cell and/or squamous cell carcinomas. As mentioned in Results at the time of sacrifice, when 60% of OH-BBN control rats had large bladder tumors, virtually all of the rats in the control or treatment groups had hyperplasias, dysplasias, or microscopic cancers. We have used the larger tumors as our primary end point because we felt that these are more likely to be similar to the clinically defined end points that are routinely used in phase III prevention trials. The use of this end point may easily identify agents that can strongly inhibit growth and progression of cancers as opposed to agents that may block the earlier parts of the carcinogenic pathway. Thus, certain of the ongoing prevention clinical trials in urinary bladder cancer are examining development of a new cancer in individuals who have had resection of a prior tumor (22). Based on these considerations, we have recently used a similar delayed administration with new agents that are being tested for chemopreventive efficacy.

Polyphenon E (a mixture of green tea polyphenols), at dose levels of 250 and 100 mg/kg BW/d, caused a dose-

dependent decrease in urinary bladder cancers. The lower dose caused a 30% decrease in the incidence of palpable tumors (control, 20 of 34; low-dose Polyphenon E, 14 of 34), whereas the higher dose decreased the incidence of palpable tumors by 70% (high dose Polyphenon E, 6 of 34;  $P < 0.01$ ). This study showed that p.o. Polyphenon E, which is nontoxic even at doses 10 times greater, is effective as a preventive agent when administered during the promotion/progression stage of carcinogenesis, and is independent of any changes in OH-BBN metabolism or the earlier stages of tumor promotion. We did not use Polyphenon E at higher nontoxic doses because it would greatly exceed a human maximal dosing. In fact, Sato et al. (13, 14) have shown that relatively high doses of dried green tea, roughly 15 to 30 g/kg diet, were effective in blocking OH-BBN-induced urinary bladder cancers. Those studies would yield gavage doses of ~1,500 and 3,000 mg/kg BW/d. In the studies of Sato et al., treatment was initiated either before OH-BBN or relatively early in the study. In contrast, our treatment was initiated 30% of the way into the study, demonstrating that the agent is effective even when given late in the carcinogenic process.

Although there was very clear efficacy of Polyphenon E in prevention of urinary bladder cancer, we observed minimal effects of this agent in the prevention of mammary cancers. Two relatively high doses of Polyphenon E (1,000 or 333 mg/kg BW/d by gavage) were used and no effect was observed on tumor incidence and only a minimal (statistically insignificant) decrease (14%) in tumor multiplicity was seen. The average weight of the cancers decreased by 30% (not statistically different). In contrast, a variety of hormonal agents (selective estrogen receptor modulators, aromatase inhibitors) and nonhormonal agents (retinoid X receptor agonists, farnesyltransferase inhibitors,

**Table 4. Serum levels of polyphenols**

Groups	(-)-Epigallocatechin	(-)-Epicatechin	(-)-Epigallocatechin gallate	(-)-Epicatechin gallate
Vehicle only	0.36 ± 0.61* (1.2 nmol/L)	0.24 ± 0.4 (0.8 nmol/L)	1.0 ± 1.1 (2.4 nmol/L)	0.2 ± 0.6 (0.7 nmol/L)
Polyphenon E, 250 mg/kg BW/d (8 h)	131 ± 33 (440 nmol/L)	741 ± 202 (2,600 nmol/L)	27 ± 9.0 (61 nmol/L)	8.1 ± 3.5 (18 nmol/L)
Polyphenon E, 250 mg/kg BW/d (16 h)	24 ± 09 (80 nmol/L)	85 ± 38 (290 nmol/L)	5.4 ± 9.1 (12 nmol/L)	7.4 ± 4.9 (15 nmol/L)

\*Values are ng/mL (and nmol/L) and are the mean ± SD obtained at termination of the study from rats not receiving the carcinogen.

and epidermal growth factor receptor inhibitors) have proven to be highly active in this specific cancer model (8, 9). The data with the epidermal growth factor receptor inhibitor are particularly interesting because it has been proposed that the tea polyphenol EGCG may work in part by inhibiting members of the epidermal growth factor receptor family (23).

When examined for levels of various polyphenols in the urine, relatively high levels of EGC and EC were found. Very low levels of EGCG, the primary polyphenol in Polyphenon E, were detected. The levels of polyphenols in urine did not decrease between 8 and 16 h after the last dose. Levels of polyphenols were 50× to 1,000× lower in serum and significantly decreased between 8 and 16 h after the last administration of Polyphenon E. Levels of M4 and M6 in serum were lower than that of the catechins and were actually below the limit of detection. These results are consistent with our previous observation that p.o. administered EGCG has very low systemic bioavailability in rats and almost all the absorbed EGCG is eliminated in the bile and excreted in feces (11, 12, 24). For unknown reasons, the bioavailability of EGCG in rats is lower than in mice or humans (12, 25, 26). Because EGCG has eight phenolic groups and a molecular weight approaching 450, its bioavailabilities in rats, mice, and humans are lower than those of EGC and EC, which have fewer phenolic groups and lower molecular weight (24, 26, 27). Therefore, substantial amounts of EGC and EC were detected in the serum samples and much higher levels of EGC and EC together with their ring fission metabolites were observed in the urine samples. Whereas EGCG is considered to be the most active tea polyphenols and has been used in most cancer prevention studies, EGC and EC also have (albeit lower) anticancer activities. It was mentioned earlier that significant levels of M4 or M6 were not seen in serum, even when compared with the relatively lower levels of EGC and EC. In contrast, M4 and M6 were seen at similar levels to these two polyphenols in urine. In this study, both the rather high urinary levels of EGC and EC (to which the bladder epithelia are directly exposed) and the systemic available polyphenols may contribute to the inhibition of bladder carcinogenesis. In the mammary tissues, however, only the rather low levels of systemic available catechins can exert the biological effects. This may explain the minimal inhibitory effect of dietary Polyphenon E on mammary tumorigenesis. Concerning the mechanisms of inhibition of carcinogenesis by tea polyphenols, most studies were conducted with EGCG (reviewed in refs. 11, 27). Although many mechanisms have been proposed, based on studies in cell lines, their clear relevance to cancer prevention in animals remains to be shown (27–29). The mechanisms of inhibition of bladder carcinogenesis by tea polyphenols need to be investigated.

This article yields a number of important findings: (a) Polyphenon E was an effective chemopreventive agent in the urinary bladder model, (b) the efficacy of Polyphenon E against bladder cancers was associated with high urinary

levels of the various tea polyphenols, and (c) Polyphenon E showed minimal efficacy in the rat mammary cancer model despite the fact that a significantly higher dose of Polyphenon E was used. This lower efficacy was associated with low levels of specific tea polyphenols in serum.

#### Acknowledgments

We thank Bonnie Mould and Tom Morgan for performing the animal studies, Julie Gray for compound purity and dietary dose verification analyses, and Mary Jo Cagle and Jeanne Hale for secretarial and editorial services.

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