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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
Our hypothesis is that PEITC is an effective preventive agent in breast cancer. Although a number of mechanisms may be involved, one mechanism of PEITC effects that has not been explored is the potential for PEITC to alter the synthesis and elimination of estrogens. Our specific aims are: (1) to evaluate the efficacy of dietary PEITC, at relevant doses, in preventing or delaying the onset of breast cancer in estrogen-dependent breast cancer animal models, (2) to evaluate the effects of PEITC on 17beta-estradiol (E_2) and metabolite (E_2 sulfate, E_2 glucuronide, estrone, 2-hydroxyE_2, 4-hydroxyE_2, 2-methoxyE_2, and 4-methoxyE_2) concentrations in plasma and tumor samples, and (3) to determine changes in enzyme activity and/or mRNA/protein expression in tumor and liver samples, for metabolic pathways which may be altered based on the E_2 and E_2 metabolite profiles. Our studies to date have characterized the pharmacokinetics of PEITC in rats (our animal model) and demonstrated in preliminary studies that PEITC can alter estrogen metabolism in the liver. Additionally, we found that low concentrations of PEITC can alter the expression of some genes important in breast cancer. These studies will provide new findings regarding the chemopreventive effects of the dietary compound PEITC in estrogen-dependent breast cancer.

14. SUBJECT TERMS
breast cancer, diet, phenethyl isothiocyanate, estrogen metabolism, organic isothiocyanates

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INTRODUCTION

Although more than 20 natural and synthetic organic isothiocyanates (ITCs) have been shown to block carcinogenesis, phenethyl isothiocyanate (PEITC), present in cruciferous vegetables, is one of the most extensively studied ITCs because of its high potency against a variety of tumors and its low *in vivo* toxicity. Our hypothesis is that PEITC is an effective preventive agent in breast cancer. Although a number of mechanisms may be involved, one mechanism of PEITC effects that has not been explored is the potential for PEITC to alter the synthesis and elimination of estrogens. Such an effect might be anticipated since PEITC can inhibit Phase I enzymes and induce Phase II enzymes. Our specific aims are: (1) to evaluate the efficacy of dietary PEITC, at relevant doses, in preventing or delaying the onset of breast cancer in estrogen-dependent breast cancer animal models, (2) to evaluate the effects of PEITC on 17beta-estradiol (E_2) and metabolite (E_2 sulfate, E_2 glucuronide, estrone, 2-hydroxyE_2, 4-hydroxyE_2, 2-methoxyE_2, and 4-methoxyE_2) concentrations in plasma and tumor samples, and (3) to determine changes in enzyme activity and/or mRNA/protein expression in tumor and liver samples, for metabolic pathways which may be altered based on the E_2 and E_2 metabolite profiles. Studies will evaluate the effects of PEITC in two estrogen-dependent animal breast cancer models (1) N-methyl-N-nitrosurea (NMU)-induced carcinogenesis in rats, and (2) estrogen-induced mammary gland cancer in ACI rats. These studies will provide new findings regarding the chemopreventive effects of the dietary compound PEITC in estrogen-dependent breast cancer.

BODY

This represents an annual report for this Concept grant, since a one year no-cost extension was granted. The no-cost extension was requested for the following reasons:
1) A research technician had to be hired, then trained, delaying the start of the research on this grant.
2) We proposed to evaluate estradiol and its metabolites in rat tissues for this grant. We found that it was necessary to have a more sensitive assay for estradiol and its metabolites, than the GC/MS assay that was proposed for use. We have modified a human radioimmunoassay for estradiol to use in rat tissues and validated its sensitivity and specificity. We are currently working on a LC/MS/MS assay to determine the low concentrations of metabolites found in tissues. This assay should allow us to evaluate the effects of phenethyl isothiocynate on estradiol metabolism in rat tissues and tumors.

Statement of Work

Task 1. To determine the dose of PEITC to be used in diets.

a. Perform preliminary pharmacokinetic studies in rats to obtain bioavailability and clearance data.
b. Have diets prepared with and without PEITC. Two diets will be prepared containing PEITC that should produce average steady state plasma concentrations of 1 μM and 10 μM.

This task has been completed, and the diets are being prepared. The manuscript on the Pharmacokinetics and Bioavailability of PEITC in rats is in preparation.

Pharmacokinetics and Bioavailability of PEITC in rats.

Purpose: Phenethyl isothiocyanate (PEITC), a dietary compound in cruciferous vegetables, has chemopreventive properties and is being investigated in Phase I clinical studies. The pharmacokinetics of PEITC are largely unknown. The objective of this study was to examine the pharmacokinetics of PEITC in rats following oral and intravenous administration.

Methods: Male Sprague-Dawley rats were administered PEITC at doses of 2, 10, 100 or 400 μmol/kg i.v. or 10, 100 or 400 μmol/kg orally. PEITC was prepared in 15% hydroxy-propyl-β-cyclodextrin. Plasma samples were collected at 5, 15, 30 min and 1, 2, 3, 6, 9, 12, 24, 36, 48, 72 and 96 h and analyzed by a LC/MS/MS assay. Pharmacokinetic data were analyzed by WinNonlin for non-compartmental analysis and ADAPT II for compartmental analysis.

Results: With an increase in the PEITC dose, elimination half-life ($t_{1/2}$) and time to C_{max} (t_{max}) increased, maximal plasma concentrations (C_{max}) increased but not proportionally, and oral bioavailability (F) decreased. At the highest dose, Cl was decreased while V was increased. The plasma concentration profile of PEITC after i.v. administration can be well characterized by a three-compartment model with Michaelis-Menten elimination and distribution.

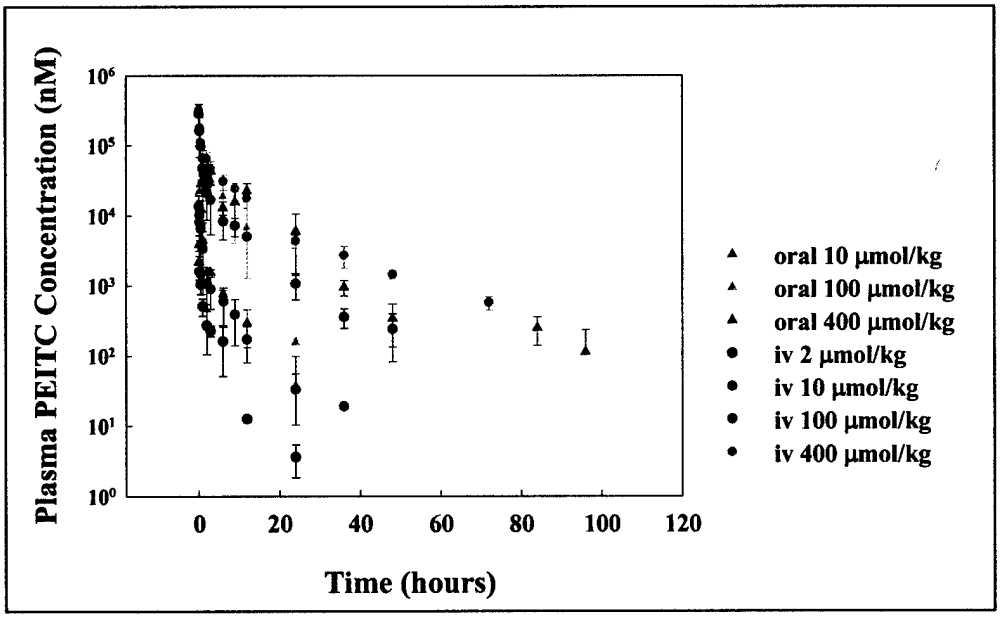

Fig. 1. The plasma concentration profile of PEITC in rats after iv and oral administration.

Table 1. Pharmacokinetic parameters of PEITC in rats

Dose (μmol/kg)	2	10	100	400
$t_{1/2}$ (h)	3.52 ± 0.35	6.92 ± 3.73	$9.19 \pm 0.83^{*}$	$13.1 \pm 2.0^{***\Delta\Delta}$
Cl (L/h/kg)	0.70 ± 0.17	0.68 ± 0.29	0.36 ± 0.18	0.50 ± 0.04
V (L/kg)	3.52 ± 0.63	7.82 ± 5.66	4.94 ± 2.84	9.46 ± 2.06
t_{max} (h)		0.4 ± 0.1	2.0 ± 1.0	$2.3 \pm 1.2^{\Delta}$
C_{max} (μM)		9.2 ± 0.6	42.1 ± 11.4	48.0 ± 5.9
F (%)		115	92.4	63.8

Abbreviations for the parameters: $t_{1/2}$, elimination half-life; Cl, clearance; V, volume of distribution; C_{max}, maximal plasma concentration; t_{max}, time to reach C_{max}; F, bioavailability. The parameters were calculated by non-compartmental analysis. Statistics were conducted by ANOVA followed by Bonferroni's test, n = 3 or 4; $^{*}P < 0.05$ and $^{***}P < 0.001$ compared to 2 μmol/kg group; $^{\Delta}P < 0.05$ and $^{\Delta\Delta}P < 0.01$ compared to 10 μmol/kg group.

Conclusions: PEITC is a dietary component with high oral bioavailability and low clearance in rats. Nonlinear elimination and distribution is evident at high doses.

Task 2. To set up assays for 17β-estradiol (E_2), estrone, 2-OHE_2, 4-OHE_2, 2-methoxyE_2 and 4-methoxyE_2. (With consultant)

a. Set up GC/MS assay for plasma samples.
b. Set up GC/MS assay for tumor tissue and mammary gland samples.
c. Validate conditions for hydrolysis of sulfate and glucuronide conjugates of E_2.

Our initial goal was to use a published GC/MS assay to measure E_2 and its metabolites. Our consultant, Dr. James Olson, had previously used this assay in studies with microsomal preparations of liver and lung isolated from rats. However, the sensitivity is low, and exogenous E_2 needs to be added to microsomal preparations. In order to detect physiological concentrations of E_2, we modified a human radioimmunoassay (Diagnostic Products, Inc.), and could detect plasma, liver and mammary gland E_2 concentrations. Since we were interested in also determining metabolite concentrations we are modifying a liquid chromatography tandem mass spectroscopy method.
We have also examined the enzymatic hydrolysis of E_2 conjugated metabolites.

A PE SCIEX API 3000 triple-quadruple tandem mass spectrometer (Applied Biosystem, Foster City, CA) equipped with a TurboIonspray(TIS) interface, a series 2000 Perkin-Elmer pump, a series 2000 Perkin-Elmer autosampler (Shelton, CT) and Analyst 1.3 software is used for data acquisition and processing. The mass spectroscopy is performed in a negative mode using multiple reaction monitoring. Optimal operating parameters of TIS were obtained, with regard to maximum signal intensity of molecular ions and fragment ions, by consecutive infusion of standard solutions of estradiol and its metabolites, using a Harvard syringe pump. The optimum conditions of the interface are as follows: ion spray voltage of -4500 V, pressure of collision gas (N_2) of 2.8mTorr, flow rate of the nebulizer gas (air) and curtain gas (N_2) of 1.2 and 0.8 L/min, respectively. The interface temperature is set at 350°C. The optimal declustering, focusing and

entrance potentials are given below. The nebulizer current is 12 μA and fragmentation is induced with a collision energy of -30eV.

Table 2 .Optimized MS Parameters:

Standards	Product Ion	Parameters
Estradiol	271.1/144.9	DP= -100 FP= -200 CE= -55
2-hydroxyestradiol	287.5/147.3	DP= -100 FP= -280 CE= -55
4- hydroxyestradiol	287.1/255.3	DP= -85 FP= -300 CE= -55
2-methoxyestradiol	301.1/286.1	DP= -55 FP= -300 CE= -40
4-methoxyestradiol	301.1/285.1	DP= -60 FP= -290 CE= -45
estrone	269.0/144.9	DP= -70 FP= -300 CE= -50

At this time, our typical limit of detection for these compounds is 5ng/ml.

Our plans are the following:

1. To increase method sensitivity , we will evaluate the use of tetraethylammonium (TEA) buffers and p-toluenesulfonhydrazide derivatization.
2. We will try to increase sensitivity by concentrating samples. preparation procedure, and then try to assay some plasma samples.

Task 3. To examine the effect of PEITC in rats with NMU-induced mammary gland tumors.

a. Three groups of rats (12-15 per group) will be treated with diets containing either no PEITC, low PEITC or high PEITC. Animals will be assessed weekly for the presence and size of mammary tumors. Latency to tumor development will be determined.
b. After about 120 days, rats are sacrificed and blood, tumor and tissues (lung, lymph nodes and liver) obtained for the evaluation of tumor number and size evaluation, and presence of metastases.
c. Determine PEITC by LC/MS/MS in plasma and tissue samples.
d. Analyze plasma and tumor samples for 17β-estradiol (E_2), E_2 following hydrolysis with sulfatase and β-glucuronidase, estrone, 2-OHE_2, 4-OHE_2, 2-methoxyE_2 and 4-methoxyE_2 using GC/MS.

These studies have not been performed. The LC/MS/MS assay for PEITC has been developed, validated and published. The LC/MS/MS assay for E_2 and its metabolites is being developed. We have set up a collaboration with Dr. Elizabeth Repasky (Roswell Park Institute) to assist with the breast cancer animal studies.

We have performed preliminary studies, examining the effects of PEITC administration on estradiol liver concentrations, and the metabolism of estradiol in rat liver microsomes (described after Task 5).

Task 4. To examine the effect of PEITC in ACI rats.

a. Three groups of rats (12-15 per group) will b treated with diets containing either no PEITC, low PEITC or high PEITC. Animals will be assessed weekly for the presence and size of mammary tumors. Latency to tumor development will be determined.
b. After about 180 days, rats are sacrificed and blood, tumor and tissues (lung, lymph nodes and liver) obtained for the evaluation of tumor number and size and presence of metastases.
d. Determine PEITC by LC/MS/MS in plasma and tissue samples
d. Analyze plasma and tumor samples for 17β-estradiol (E_2), E_2 following hydrolysis with sulfatase and β-glucuronidase, estrone, 2-OHE_2, 4-OHE_2, 2-methoxyE_2 and 4-methoxyE_2 using GC/MS.

These studies have not been performed. The LC/MS/MS assay for PEITC has been developed, validated and published. The LC/MS/MS assay for E2 and its metabolites is being developed. We have set up a collaboration with Dr. Elizabeth Repasky (Roswell Park Institute) to assist with the breast cancer animal studies.

Task 5. To determine changes in enzyme activity, mRNA and protein expression for E_2 metabolic pathways which may be altered, based on the E_2 and E_2 metabolite profile.

a. The enzymes of interest include: aromatase, cytochrome P450(CYP)1A1/2, CYP1B, and various conjugating enzymes (sulfotransferase, UDP-glucuronyltransferase, catechol O-methyltransferase). Sulfatase and β-glucuronidase will also be examined. Activity levels will be determined in tumor and/or liver, using standard assays.
b. mRNA levels will be determined using a specific metabolic cDNA array from Superarray, so that many enzymes may be screened.
c. Protein expression will be examined using specific antibodies and Western analysis.

This task is approximately 70% complete. We have examined the effects of low PEITC concentrations in 2 different cDNA arrays available from Superarray Inc.: 1) Metabolizing Enzymes and 2) Breast cancer genes.

a) Effect of oral PEITC treatment on liver estradiol concentrations in rats.

Preliminary studies were conducted in female Sprague Dawley rats fed 400μmol/kg PEITC (in 15% hydroxypropyl-beta-cyclodextrin) twice daily for 5 days by oral gavage. Animals were sacrificed and blood and liver removed for estradiol analysis by a modified RIA (Diagnostic Products). Physiological concentrations of 17β-estradiol were decreased in livers of treated animals (Fig. 2), and the rate of estradiol metabolism was increased in liver microsomes of rats treated with oral PEITC (Table 3). **These findings support our hypothesis that PEITC can alter the metabolism of estrogens.**

Fig. 2

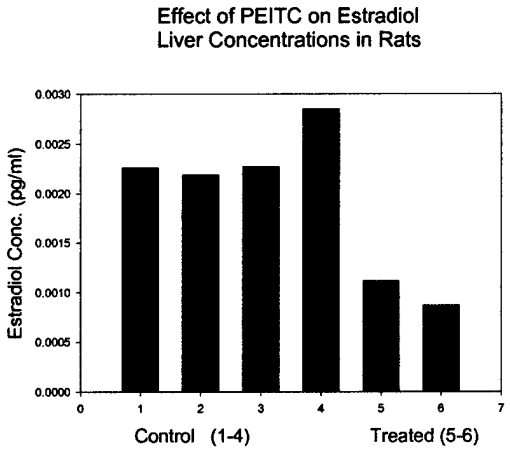

Table 3. Rate of metabolism of 17β-estradiol in rat liver microsomes. Significantly different, n=4, p<0.005

	Control mean ±SD	Treated with PEITC mean ±SD
Rate of metabolism (pmol/mg/min)	94.6 ± 2.3	128 ± 7.0

b) **Effects of PEITC (3 μM) treatment on Drug Metabolizing Enzymes in Human Hepatocytes**. We chose to determine the potential effect of PEITC on metabolizing enzymes by evaluating the effect of a 5-day incubation with PEITC (3 μM) on the gene expression in human hepatocytes (In Vitro Technologies). This technique allowed us to examine a wide range of enzymes. We used the GEArray® Q series Human Drug Metabolism Superarrys containing 96 genes encoding for drug metabolizing enzymes. Briefly, cDNA probes were synthesized by reverse transcription using 1 μg of the control (vehicle treatment) or treated RNA samples as the templates and labeled with biotin-16-dUTP (Roche). The cDNA probes are then denatured and hybridized with GEArray® membranes. The hybridization signal was detected with Kodak Image Station. and the relative abundance of a particular transcript was normalized against the signal of glyceraldehyde 3 phosphate dehydrogenase (GAPDH). The differences between the control and treated RNA samples were evaluated by significant analysis of microarrays (SAM) and student's t-test. There were no significant differences detected. This may reflect the variability observed with the human hepatocyte preparations.

Conclusions: At a concentration of 3 μM of PEITC, we did not observe any significant changes in mRNA expression of drug metabolizing enzymes. We would like to repeat this with rat liver samples, and human breast cancer cell lines. Additionally, these results do not rule out direct inhibition of metabolizing enzymes.

c) **Effects of PEITC Treatment on estrogen receptor signaling in human mammary epithelial cells (HMEC) and human breast cancer MCF-7 cells**

Methods: HMEC cells were obtained from Dr. Martha Stampfer (Lawrence Berkeley National Laboratory) and were incubated until 60-80% confluence at 1% CO2 in a 37oC incubator. MCF-7 cells were incubated until 60-80% confluence at 5% CO2 in a 37°C incubator. HMEC and MCF-7 cells were treated with DMSO (control) or PEITC at one of two concentrations (0.3 or 3.0 μM) for 48hrs. One of two methods was used to harvest cells from 75ml flask for RNA isolation.

MCF-7- all harvested via ice cold 1XPBS cell scrapping method (1XPBS-SC)

HMEC- 0.3uM samples harvested by 1X PBS-SC, 3uM via direct application of lysis buffer (Promega SV Total RNA Isolation Kit)

Total RNA was isolated from both cell lines by using the SV RNA Isolation System (Promega, Madison WI). RNA was determined spectrophotometrically at 260 nm. (0.5mg/ml-

5mg/ml are required for the gene array). GEArray Q series Estrogen Receptor Signaling Gene Array kit (SuperArray Inc., Bethesda, MD) were used, according to the manufacturer's instructions. cDNA was prepared from total RNA by reverse transcription with MMLV reverse transcriptase (Invitrogen , Buffalo, NY). Ninety-six genes were used to study the expression profile of the genes involved in estrogen receptor signaling pathways. Spots were detected using chemiluminescence-labeled using biotin (Invitrogen, Buffalo, NY), then hybridized under precisely specified conditions to a positively charged nylon membrane containing the arrayed DNA. After washing, the relative expression level of each gene was analyzed using a Kodak Image Station 440CF.

Data was analyzed using ROI analysis which measures the mean intensity for each gene spot. The background (PUC18/Blank) was subtracted from the spot net mean intensity. The housekeeping genes, β-actin and glyceraldehyde 3 phosphate dehydrogenase (GAPDH),as well as the average of total intensity, were used for normalization. Statistical analysis used the program SAM (Significance Analysis of Microarrays), which is a microarray data analysis package used to correct for the high false-positive rates due to multiple comparisons.

Table 4. Effect of Incubations with 3μM PEITC on Gene Expression in MCF-7 and HMEC Cells

Cells	Gene	PEITC-treated/Control
MCF-7	EGRF	3.23
HMEC	C3	4.00
HMEC	MLP	4.51
HMEC	TNFAIP2	0.48

EGRF = epidermal growth factor receptor (breast cancer prognosis)
C3 = complement component 3 (associated with estrogen receptor signaling)
MLP = MARCKS-like protein
TNFAIP2 = tumor necrosis factor, alpha-induced protein 2 (directly associated with breast cancer)

Table 5. Effect of Incubations with 0.3 μM PEITC on Gene Expression in MCF-7 Cells

Gene	PEITC-treated /Control
IL6	0.45
IL6R	0.69
SERPINE1	5.94
TNFRSF6	0.15

IL6 = interleukin 6 (associated with breast cancer prognosis)
IL6R = interleukin 6 receptor (associated with breast cancer prognosis)
SERPINE1 = serine (or cysteine) proteinase inhibitor (associated with breast cancer prognosis)
TNFRSF6 = tumor necrosis factor receptor superfamily, member 6

Conclusions: The studies are on-going, but interestingly, showed differences between the 2 low concentrations of PEITC used. These changes need to be confirmed, and the different effects of PEITC on normal mammary cells and breast cancer cells examined. A number of the changes appear to be important in cell cycle regulation and apoptosis.

Other

Review Paper.

We are writing a review on **Isothiocyanates and Cancer Prevention** for a book entitled Nutrition and Cancer Prevention. This is in progress, with a deadline of October 1, 2004.

KEY RESEARCH ACCOMPLISHMENTS-2003-04

- The pharmacokinetics of PEITC have been determined in rats. We have shown for the first time that the clearance of PEITC is dose dependent, and that following oral administration of PEITC, the bioavailability is high. Due to the high systemic availability of PEITC, it can be administered in the diet.
- Our preliminary data support our hypothesis that PEITC alters estradiol concentrations due to changes in metabolism, and low concentrations can result in altered gene expression.

REPORTABLE OUTCOMES

Manuscripts: Chapter entitled **Isothiocyanates and Cancer Prevention** is under preparation for the book Nutrition and Cancer Prevention.

Abstracts (in Appendix):

The Pharmacokinetics of Phenethyl Isothiocyanate (PEITC) in Rats. Ji Y and Morris ME, to be presented at the American Association of Pharmaceutical Scientists Meeting, Baltimore MD, November 2004.

Graduate Students Participating in this Research as a part of their educational program during 2003-04:

Yan Ji, Ph.D. candidate (degree expected, 2005)
Urvi Telang, Ph.D. candidate (degree expected, 2008)

Professional student Participating in the Research as a part of her educational program during 2003-04:

Elizabeth Scott-Ramsay (PharmD awarded, May 2004)

Research technician: Yang Qu

CONCLUSIONS

1. The pharmacokinetics and bioavailability of PEITC were determined in rats. The clearance of PEITC was dose dependent, but PEITC exhibited excellent bioavailability following oral administration.
2. Our preliminary results show an effect of PEITC on the concentrations and metabolism of estradiol in rat liver.

Appendix

The Pharmacokinetics of Phenethyl Isothiocyanate (PEITC) in Rats
Ji Y and Morris ME. Department of Pharmaceutical Sciences, University at Buffalo, Amherst NY 14260.

Purpose: Phenethyl isothiocyanate (PEITC), a dietary compound in cruciferous vegetables, has chemopreventive properties and is being investigated in Phase I clinical studies. The pharmacokinetics of PEITC are largely unknown. The objective of this study was to examine the pharmacokinetics of PEITC in rats following oral and intravenous administration.

Methods: Male Sprague-Dawley rats were administered PEITC at doses of 2, 10, 100 or 400 μmol/kg i.v. or 10, 100 or 400 μmol/kg orally. PEITC was prepared in 15% hydroxy-propyl-β-cyclodextrin. Plasma samples were collected at 5, 15, 30 min and 1, 2, 3, 6, 9, 12, 24, 36, 48, 72 and 96 h and analyzed by a LC/MS/MS assay. Pharmacokinetic data were analyzed by WinNonlin for non-compartmental analysis and ADAPT II for compartmental analysis.

Results: With an increase in the PEITC dose, elimination half-life ($t_{1/2}$) and time to C_{max} (t_{max}) increased, maximal plasma concentrations (C_{max}) increased but not proportionally, and oral bioavailability (F) decreased. At the highest dose, Cl was decreased while V was increased. The plasma concentration profile of PEITC after i.v. administration can be well characterized by a three-compartment model with Michaelis-Menten elimination and distribution.

Dose (μmol/kg)	2	10	100	400
$t_{1/2}$ (h)	3.52 ± 0.35	6.92 ± 3.73	9.19 ± 0.83	13.1 ± 2.0
Cl (L/h/kg)	0.70 ± 0.17	0.68 ± 0.29	0.36 ± 0.18	0.50 ± 0.04
V (L/kg)	3.52 ± 0.63	7.82 ± 5.66	4.94 ± 2.84	9.46 ± 2.06
C_{max} (μM)		9.2 ± 0.6	42.1 ± 11.4	48.0 ± 5.9
t_{max} (h)		0.4 ± 0.1	2.0 ± 1.0	2.3 ± 1.2
F (%)		115	92.4	63.8

Conclusions: PEITC is a dietary component with high oral bioavailability and low clearance in rats. Nonlinear elimination and distribution is evident at high doses.
Support by Contract DAMD17-00-1-0376 and 17-03-1-0527.

Accepted for presentation at the American Association of Pharmaceutical Scientists Annual Meeting, Baltimore MD, October 2004.