

# Effects of Cruciferous Vegetable Consumption on Urinary Metabolites of the Tobacco-Specific Lung Carcinogen 4-(Methylnitrosamino)-1-(3-Pyridyl)-1-Butanone in Singapore Chinese

Stephen S. Hecht,<sup>1</sup> Steven G. Carmella,<sup>1</sup> Patrick M.J. Kenney,<sup>1</sup> Siew-Hong Low,<sup>2</sup> Kazuko Arakawa,<sup>3</sup> and Mimi C. Yu<sup>3</sup>

<sup>1</sup>University of Minnesota Cancer Center, Minneapolis, Minnesota; <sup>2</sup>Department of Community, Occupational, and Family Medicine, National University of Singapore, Singapore; and <sup>3</sup>Norris Comprehensive Cancer Center, University of Southern California, Los Angeles, California

## Abstract

Vegetable consumption, including cruciferous vegetables, is protective against lung cancer, but the mechanisms are poorly understood. The purpose of this study was to investigate the effects of cruciferous vegetable consumption on the metabolism of the tobacco-specific lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in smokers. The study was carried out in Singapore Chinese, whose mean daily intake of cruciferous vegetables is three times greater than that of people in the United States. Eighty-four smokers provided urine samples and were interviewed about dietary habits using a structured questionnaire, which included questions on consumption of nine commonly consumed cruciferous vegetables. Samples of these vegetables obtained in Singapore markets at three different times of year were analyzed for glucosinolates. Urine was analyzed for metabolites of NNK: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and its glucuronides (NNAL-Glucs). Glucobrassicins, which release indole-3-carbinols on chewing, were the

major glucosinolates in seven of the nine cruciferous vegetables, accounting for 70.0% to 93.2% of all glucosinolates in these vegetables. There was a significant correlation ( $P = 0.01$ ) between increased consumption of glucobrassicins and decreased levels of NNAL in urine after adjustment for number of cigarettes smoked per day; similar trends were observed for NNAL-Glucs ( $P = 0.08$ ) and NNAL plus NNAL-Glucs ( $P = 0.03$ ). These results are consistent with those of previous studies, which demonstrate that indole-3-carbinol decreases levels of urinary NNAL probably by inducing hepatic metabolism of NNK. The results are discussed with respect to the known chemopreventive activity of indole-3-carbinol against lung tumorigenesis by NNK in mice and the effects of isothiocyanates, which are also formed on consumption of cruciferous vegetables, on NNK metabolism. The results of this study demonstrate the complexities in assessing effects of cruciferous vegetables on carcinogen metabolism. (Cancer Epidemiol Biomarkers Prev 2004;13(6):997–1004)

## Introduction

Epidemiologic studies demonstrate with remarkable consistency that vegetable consumption decreases the risk for lung cancer (1). An evaluation published in 1997 concluded that “the evidence that diets high in vegetables and fruits decrease the risk of lung cancer is convincing” (2). Studies published since then continue to support this conclusion (3–12), although an IARC working group evaluated the evidence as “limited” (13). Several studies of cruciferous vegetable consumption in particular and lung cancer risk also show protective effects (14).

A unique characteristic of cruciferous vegetables is their relatively high concentration of glucosinolates

(15–18). These plant defense compounds are present in milligram quantities in normal servings of cruciferous vegetables. On consumption of the raw vegetables, the plant enzyme myrosinase is released and catalyzes the hydrolysis of the glucosinolates. Glucosinolates in cooked vegetables are also hydrolyzed, but to a lesser extent, by gut bacterial myrosinase (19, 20). The hydrolysis products depend on the structure of the glucosinolate. Alkyl and aralkyl glucosinolates yield mainly isothiocyanates (ITCs) on myrosinase-catalyzed hydrolysis, whereas indolyl glucosinolates (glucobrassicins) yield predominantly indole-3-carbinol or related substituted indole-3-carbinols (15–18). ITCs and indole-3-carbinol are chemopreventive agents against carcinogenesis of the lung and other tissues in laboratory animals and exert protective effects such as inhibition of carcinogen activating enzymes, enhancement of carcinogen detoxifying enzymes, and induction of apoptosis (21–26). However, indole-3-carbinol is also a liver tumor promoter in animal models (23). The chemopreventive properties of ITCs and indole-3-carbinol provide a rationale for the results of the epidemiologic studies

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**Requests for reprints:** Stephen S. Hecht, University of Minnesota Cancer Center, Mayo Mail Code 806, 420 Delaware St. SE, Minneapolis, MN 55455. Phone: 612-624-7604; Fax: 612-626-5135. E-mail: hecht002@umn.edu

cited above. Consistent with these data, three epidemiologic studies demonstrate that ITCs are protective against lung cancer, particularly in the presence of *GSTM1/T1* null genotypes (27-29).

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is a tobacco-specific lung carcinogen that is believed to play a significant role as a cause of lung cancer in smokers (30, 31). NNK is a procarcinogen that requires metabolism to exert its carcinogenic effects. An overview of NNK metabolism is presented in Fig. 1 (32). Oxidative metabolism of NNK by  $\alpha$ -hydroxylation, catalyzed by cytochrome P450 enzymes such as P450s 1A2 and 2A13, results in the formation of DNA adducts in the lung and other tissues, leading to permanent mutations and tumors. Reductive metabolism of NNK gives 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), which, like NNK, is a potent lung carcinogen in rats and mice and is metabolically activated by  $\alpha$ -hydroxylation. NNAL also can undergo glucuronidation at the pyridine nitrogen and hydroxyl oxygen with formation of the non-carcinogenic detoxification products NNAL-Glucs (32, 33). Glucuronidation of NNAL is catalyzed by UDP-glucuronosyltransferases such as UGT 1A4, 1A9, and 2B7 (34). NNAL and NNAL-Glucs are readily quantifiable in human urine (35).

Indole-3-carbinol and 2-phenethyl ITC (PEITC) are two specific glucosinolate hydrolysis products that can prevent lung tumor induction by NNK in rodents. Indole-3-carbinol inhibits NNK-induced lung tumorigenesis in A/J mice (36). Mechanistic studies demonstrate that indole-3-carbinol induces hepatic  $\alpha$ -hydroxylation of NNK in mice, thus decreasing NNK dose to the lung (36). The increased hepatic  $\alpha$ -hydroxylation diverts NNK from the metabolic reduction pathway, thus resulting in decreased excretion of NNAL and NNAL-Glucs in the urine of these mice (Fig. 1; ref. 36). In smokers treated with indole-3-carbinol, significant decreases of NNAL and NNAL plus NNAL-Glucs (total NNAL) in urine were also observed, consistent with the observations in mice (37). PEITC inhibits NNK-induced lung tumorigenesis in rats and A/J mice (38-41). The major mechanism appears to be inhibition of  $\alpha$ -hydroxylation and DNA adduct formation by NNK in the lung (40, 42-44). In rats, this clearly results in shunting of more NNK to the metabolic reduction pathway, thereby increasing excretion of NNAL and NNAL-Glucs in urine (Fig. 1; refs. 40, 44). In smokers who consumed watercress, a source

of abundant PEITC via hydrolysis of its glucosinolate precursor gluconasturtiin, increased levels of NNAL and NNAL-Glucs were observed in urine, consistent with the rat data (45).

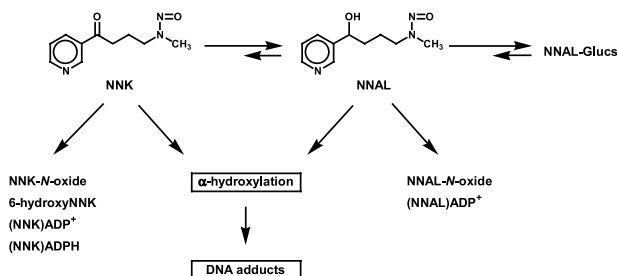
Therefore, the effects of vegetable consumption on NNK metabolism can be complex and contradictory depending on the situation. Consumption of vegetables rich in glucobrassicins would be expected to yield significant amounts of indole-3-carbinols, which should diminish levels of NNAL or NNAL-Glucs in urine. Consumption of vegetables such as watercress, rich in gluconasturtiin, the glucosinolate precursor to PEITC, would be expected to increase levels of NNAL and NNAL-Glucs in urine. In this study, we measured NNAL and NNAL-Glucs in the urine of Chinese smokers in Singapore. Singapore Chinese consume a diet rich in cruciferous vegetables (mean consumption, 345 times per year; mean daily intake, 40.6 g), more than three times the level of intake in the United States (46). The effects of cruciferous vegetable constituents on levels of NNAL and NNAL-Glucs in urine were assessed.

## Subjects and Methods

**Study Population.** The subjects were participants in the Singapore Chinese Health Study, a population-based, prospective investigation of diet and cancer risk (47). Briefly, 63,257 Chinese women and men from two major dialect groups in Singapore (Hokkien and Cantonese) were recruited between April 1993 and December 1998. Subjects were between ages 45 and 74 and resided in government housing estates. Eighty-six percent of the Singapore population resided in such facilities during the period of study enrollment.

In April 1994, we began collecting blood (or buccal cells) and single-void urine specimens from a random 3% sample of study participants. Accrual of this biospecimen subcohort was extended to all surviving cohort participants beginning in January 2000. Details of the collection, processing, and storage of the spot urine samples have been described (46). Briefly, the urine specimens were kept on ice immediately after collection and processed (acidified with 2 g of ascorbic acid per 100 mL of urine) within 1 h. The specimens remained frozen at  $-80^{\circ}\text{C}$  until air transportation on dry ice from Singapore to the University of Minnesota Cancer Center. Subjects in the present study were the first 84 participants in the biospecimen subcohort, who were current cigarette smokers at baseline. The study protocol was approved by the institutional review boards of the University of Southern California and the National University of Singapore.

**Baseline Questionnaire Data.** At recruitment, a face-to-face interview was conducted in the home of the subject by a trained interviewer using a structured questionnaire focusing on dietary habits during the past 12 months. The validated, 165-item semiquantitative food frequency questionnaire included nine commonly consumed cruciferous vegetables in a typical Singapore Chinese diet (see below). Previously, we used a cycl-condensation assay to determine the total ITC contents of market samples of these nine cruciferous vegetables



**Figure 1.** Overview of NNK metabolism. For further details, see ref. (31).

and computed daily intake levels of total ITC in study subjects via linkage of total ITC contents in vegetables with responses to the dietary questionnaire administered at baseline. Using the same cyclocondensation assay, we also measured levels of total ITC in spot urine collected from 246 cohort subjects and demonstrated a close and statistically significant association between dietary and urinary total ITC levels among these subjects (46). In the present study, contents of individual glucosinolates in market samples of the nine cruciferous vegetables consumed by Singapore Chinese were measured (see below). We calculated the daily intake levels of total glucobrassicins by summing the intake levels of glucobrassicin, 4-hydroxyglucobrassicin, 4-methoxyglucobrassicin, and 1-methoxyglucobrassicin. Similarly, we calculated the daily intake levels of total ITC by summing intake levels of the remaining glucosinolates listed in Table 1.

The questionnaire also requested information on lifetime use of cigarettes. Ever smokers were asked about the age he/she began smoking on a regular basis, total years he/she smoked on a regular basis, and average number of cigarettes smoked per day, and ex-smokers were asked about the number of years since he/she stopped smoking. For average number of cigarettes smoked per day, subjects were given six categories to choose from:  $\leq 6$ , 7 to 12, 13 to 22, 23 to 32, 33 to 42, and  $\geq 43$ .

#### Collection and Processing of Vegetable Samples.

Three samples of each of nine types of cruciferous vegetables were purchased from various markets in Singapore on three different dates: October 12, 1999; January 11, 2000; and April 11, 2000. The vegetables were broccoli (*Brassica oleracea italica*), cabbage (*B. oleracea capitata*), cauliflower (*B. oleracea botrytis*), choy sum (*Brassica chinensis parachinensis*, also known as Chinese flowering cabbage), kai choy (*Brassica juncea rugosa*, also known as mustard cabbage or Chinese mustard), kai lan (*Brassica alboglabra*, also known as Chinese kale), pak choy (*B. chinensis*, also known as Chinese white cabbage), watercress (*Nasturtium officinale*), and wong nga pak (*Brassica pekinensis cylindrica*, also known as celery cabbage). Each sample (200 g) was cooked in boiling water for 3 minutes on the day of purchase. The cooked vegetables were sealed in plastic bags, and 50 mL of the 500 mL of cooking water were placed in a plastic centrifuge tube. The samples were then frozen and shipped by air carrier to the University of Minnesota Cancer Center.

**Analysis of Glucosinolates in Vegetables.** The vegetables were divided into three aliquots and each was weighed. One portion was placed in  $\sim 200$  mL  $H_2O$  and the mixture was homogenized for 2 minutes in a food blender. The other two portions were stored at  $-20^\circ C$  for future analyses. A 5 mL aliquot of the vegetable suspension was placed in a 15 mL conical tube and homogenized with a Janke-Kundel Ultra Turrax T23 homogenizer (Fisher Scientific, Pittsburgh, PA) at  $12,000 \times g$  for 2 minutes at  $4^\circ C$ . The homogenate was centrifuged at  $2,000 \times g$  for 15 minutes at  $4^\circ C$ . The supernatant was removed and assayed for glucosinolates by a modification of a previously described method for analysis of desulphoglucosinolates (48). Strong anion

exchange solid-phase extraction cartridges (500 mg, E. Merck, VWR Scientific Products catalogue EM-2025-1, West Chester, PA) were mounted in a 16 port manifold and conditioned with 2 mL of 0.5 mmol/L sodium acetate buffer (pH 4.6). They were then washed with 2 mL of deionized  $H_2O$ . Aliquots (500  $\mu$ l) of the supernatants from above were applied to the strong anion exchange columns and the columns were washed with 1 mL of 0.2 mmol/L sodium acetate buffer (pH 4.0). Then, 1 mL (3 units) of sulfatase solution (Sigma S-9626, Sigma-Aldrich, St. Louis, MO) was applied to the strong anion exchange columns. The enzyme solution was allowed to flow through the columns until the meniscus reached the top of the packing. The columns containing the enzyme were allowed to stand overnight at room temperature to release the desulphoglucosinolates. Then,  $H_2O$  (3 mL) was applied to elute the desulphoglucosinolates. The eluants were placed in 2 mL autosampler vials. The elution volume was determined by weight and 0.1 mL aliquots were analyzed by high-performance liquid chromatography. We used a  $250 \times 4.6$  mm Luna C18 5  $\mu$ m column (Phenomenex, Torrance, CA) attached to a C18 guard column. Detection of desulphoglucosinolates was accomplished with a SPD-10AV UV-visible detector operated at 229 nm (Shimadzu, Columbia, MD). The linear gradient program was as follows: 5% to 15% acetonitrile in  $H_2O$  for 2 minutes, then 15% to 65% for 28 minutes, then hold for 5 minutes, then return to 5% acetonitrile in  $H_2O$  for 2 minutes, then hold for 23 minutes at a flow rate of 1 mL/min. Calibration curves were constructed using known amounts of desulphosinigrin, which was prepared from sinigrin (Sigma-Aldrich) as described above. The other glucosinolates were identified by their retention times using standards kindly provided by Dr. Richard Mithen (Institute of Food Research, Norwich, United Kingdom) and quantified using known response factors as reported (49). Aliquots of the cooking water were directly applied to the conditioned strong anion exchange columns and analyzed the same way, and the amounts were added to those found in the cooked vegetables.

**Analysis of NNAL and NNAL-Glucs in Urine.** This was carried out as described (50). The method involves extraction of urine with organic solvents, high-performance liquid chromatography purification, and quantitation by gas chromatography with nitrosamine selective detection. Creatinine was assayed by Fairview-University Medical Center Diagnostic Laboratories (Minneapolis, MN) using Vitros CREA slides.

**Statistical Analysis.** The distributions of urinary NNAL and NNAL-Glucs in our study population were markedly skewed; therefore, formal statistical testing was performed on logarithmically transformed values of urinary NNAL, NNAL-Glucs, and total NNAL, and geometric (as opposed to arithmetic) mean values are presented. The ANOVA method (51) was used to examine the effects of smoking intensity (number of cigarettes smoked per day) and dietary glucobrassicins simultaneously on urinary levels of NNAL, NNAL-Glucs, and total NNAL. Among study subjects, the nonparametric Spearman correlation coefficient was used to examine the correlations among dietary total ITC determined by the cyclocondensation assay (46),

**Table 1. Glucosinolates in vegetables purchased in Singapore**

Compound*	Vegetable		
	Broccoli	Pak Choi	Wong Nga Pak
Glucoraphanin	522 ± 404	NQ	NQ
Gluconasturtiin	NQ	NQ	3.5 ± 6.1
Sinigrin	NQ	NQ	NQ
Glucobrassicin	4,250 ± 1,060	264 ± 79.9	839 ± 912
4-Hydroxyglucobrassicin	198 ± 139	158 ± 29.8	84.4 ± 146
4-Methoxyglucobrassicin	432 ± 79.4	256 ± 89.8	1,810 ± 701
1-Methoxyglucobrassicin	1,100 ± 186	113 ± 31.3	1,360 ± 1,560
Glucoiberin	40.9 ± 42.4	16.0 ± 7.8	NQ
Progoitrin (or epiprogoitrin)	NQ	37.8 ± 21.6	73.0 ± 43.0
Glucoalyssin	18.4 ± 16.4	15.6 ± 9.6	77.1 ± 62.3
Gluconapoleiferin	NQ	NQ	9.3 ± 16.0
Gluconapin	NQ	158 ± 100	197 ± 254
Glucobrassicinapin	NQ	52.3 ± 38.1	192 ± 239
Glucoerucin	NQ	NQ	NQ
7-Methylthioheptyl	NQ	NQ	NQ
8-Methylthiooctyl	NQ	NQ	NQ
Total glucosinolates (nmol/g wet weight)	6,570 ± 1,290	1,072 ± 272	4,640 ± 2,970
Glucobrassicins (% of total glucosinolates)	91.0	73.7	70.0

NOTE: Values are mean ± SD ( $n = 3$ ) of amounts (nmol glucosinolate per gram wet weight) in vegetables purchased at markets in Singapore at three different times of the year. Values include amounts in vegetable plus cooking water. Amounts in cooking water (% of total) were broccoli, 53; pak choi, 20; wong nga pak, 26; watercress, 33; kai choi, 22; kai lan, 29; choi sum, 33; cauliflower, 31; and cabbage, 37. NQ, not quantified. Limit of quantitation, 10 nmol/g wet weight. Some values are below 10 nmol/g wet weight because data from three different vegetable purchases were averaged.

\*Identified by comparison of retention times and UV spectra to those standards.

dietary total glucobrassicins, and dietary total ITC derived from levels of glucosinolate precursors. All  $P$  values quoted are two sided.  $P < 0.05$  is considered statistically significant.

## Results

By design, all study subjects (74 males and 10 females) were cigarette smokers at the time of recruitment to the cohort study, which was 1 to 2 years prior to time of urine collection. The mean (SD) age of the subjects at urine collection was 59.0 (7.5) years. The mean (SD) self-reported number of cigarettes smoked per day by the subjects was 19.3 (11.1). The geometric mean (95% confidence interval) level and range of NNAL in urine were 0.51 (0.41-0.61) and 0 to 3.62 pmol/mg creatinine, respectively. The corresponding figures for urinary NNAL-Glucs were 1.11 (0.91-1.32) and 0 to 5.07 pmol/mg creatinine. The geometric mean (95% confidence interval) level of the NNAL-Glucs/NNAL ratio was 2.39 (1.99-2.85). The range of this latter ratio was 0 to 39.6. Levels of NNAL, NNAL-Glucs, and total NNAL showed statistically significant, dose-dependent associations with intensity of smoking (Table 2).

The structures of the glucosinolates found in the vegetables are shown in Fig. 2. Glucobrassicins (glucobrassicin, 1-methoxyglucobrassicin, 4-methoxyglucobrassicin, and 4-hydroxyglucobrassicin), precursors to indole-3-carbinols, were the predominant glucosinolates in seven of the nine vegetables studied, accounting for 70.0% to 93.2% of all glucosinolates detected by our method in these vegetables (Table 1). The only vegetables in which glucobrassicins were not the major glucosinolates were watercress, in which gluconasturtiin accounted for 28% of all glucosinolates, and kai choi, in which sinigrin accounted for 71% of all glucosinolates. Other glucosinolates found in substantial quantities (>100 nmol/g

wet weight) were glucoraphanin (broccoli), glucoiberin (cabbage), gluconapin (pak choi, wong nga pak, and kai lan), glucobrassicinapin (wong nga pak), and 7-methylthioheptyl glucosinolate (watercress).

Glucosinolate precursors to ITCs with known chemopreventive activity were glucoraphanin (sulforaphane), gluconasturtiin (PEITC), and glucotropaeolin (benzyl ITC; ref. 21). Substantial quantities of glucoraphanin and gluconasturtiin were found in broccoli and watercress, respectively, but only small amounts elsewhere. Glucotropaeolin was not detected in the vegetables studied here.

As expected, dietary glucobrassicins, the two indices of dietary total ITC, and intake of total cruciferous vegetables were highly correlated variables among the study subjects. All pairwise correlation coefficients were at least 0.88 and all were highly statistically significant ( $P < 0.0001$ ).

The effects of glucobrassicin consumption and cigarette smoking, with adjustment for each other, on the levels of NNAL, NNAL-Glucs, and total NNAL in urine are summarized in Table 2. There was a significant association between increasing glucobrassicin intake and decreasing levels of free NNAL ( $P = 0.01$ ). Levels of NNAL-Glucs and total NNAL also were lower at the two highest versus lower levels of glucobrassicin intake, with the association either of borderline statistical significance (NNAL-Glucs) or achieving statistical significance (total NNAL). Similar but generally weaker relationships were observed between dietary ITC (either of the two indices) or total cruciferous vegetable intake and urinary NNAL, NNAL-Glucs, and total NNAL (data not shown).

During cooking, 20% to 53% of the glucosinolates were extracted into the cooking water (Table 2). The results of the analysis presented above were essentially the same whether the total amounts of glucosinolates or only those remaining in the vegetables were used.

**Table 1. Glucosinolates in vegetables purchased in Singapore (Cont'd)**

Vegetable					
Watercress	Kai Choi	Kai Lan	Choi Sum	Cauliflower	Cabbage
NQ	NQ	51.7 ± 48.7	NQ	NQ	NQ
1,010 ± 432	4.5 ± 7.8	NQ	NQ	NQ	NQ
NQ	2,100 ± 1,110	64.0 ± 21.7	NQ	132 ± 95.6	207 ± 109
1,165 ± 344	47.4 ± 15.1	491 ± 97.4	75.3 ± 32.1	2,850 ± 1,950	3,550 ± 2,230
NQ	247 ± 201	107 ± 16.7	NQ	75.8 ± 22.3	23.3 ± 25.9
674 ± 94	203 ± 44.8	752 ± 522	295 ± 52.7	231 ± 61.0	691 ± 80.2
NQ	251 ± 136	485 ± 332	114 ± 95.0	743 ± 725	124 ± 63.3
NQ	NQ	26.8 ± 10.5	NQ	NQ	191 ± 147
NQ	NQ	51.9 ± 57.7	46.9 ± 12.8	NQ	NQ
NQ	NQ	NQ	NQ	14.1 ± 12.2	NQ
NQ	NQ	13.4 ± 13.9	NQ	NQ	NQ
22.5 ± 23.7	74.2 ± 31.7	369 ± 49.9	83.6 ± 10.4	NQ	65.3 ± 94.6
NQ	NQ	NQ	27.6 ± 6.8	NQ	NQ
77.3 ± 52.5	NQ	NQ	NQ	NQ	NQ
218 ± 60.6	NQ	NQ	NQ	NQ	NQ
8.1 ± 3.4	NQ	NQ	NQ	NQ	NQ
3,640 ± 1,310	2,950 ± 1,370	2,410 ± 409	654 ± 184	4,190 ± 2,650	5,000 ± 2,560
47.9	25.3	76.0	74.1	93.2	87.8

## Discussion

Levels of NNAL and NNAL-Glucs measured in the urine of our subjects were similar to those reported in previous studies of smokers, carried out in mainly Caucasian populations (35). Ratios of NNAL-Glucs/NNAL were also similar to those in previous studies. We observed significant correlations between NNAL, NNAL-Glucs, and total NNAL and number of cigarettes smoked per day. One previous study reported a correlation between total NNAL and number of cigarettes smoked per day in African Americans but not Caucasians (52), while another found no relationship (53). Because NNAL and NNAL-Glucs are metabolites of a tobacco-specific compound, NNK, correlations with cigarettes per day would be expected in the absence of other factors. However, interindividual differences in NNK metabolism and in the way cigarettes are smoked will affect the strength of the relationship between urinary total NNAL and cigarettes per day (54).

The literature on glucosinolates in plants is extensive (15-18). Our data are generally consistent with published

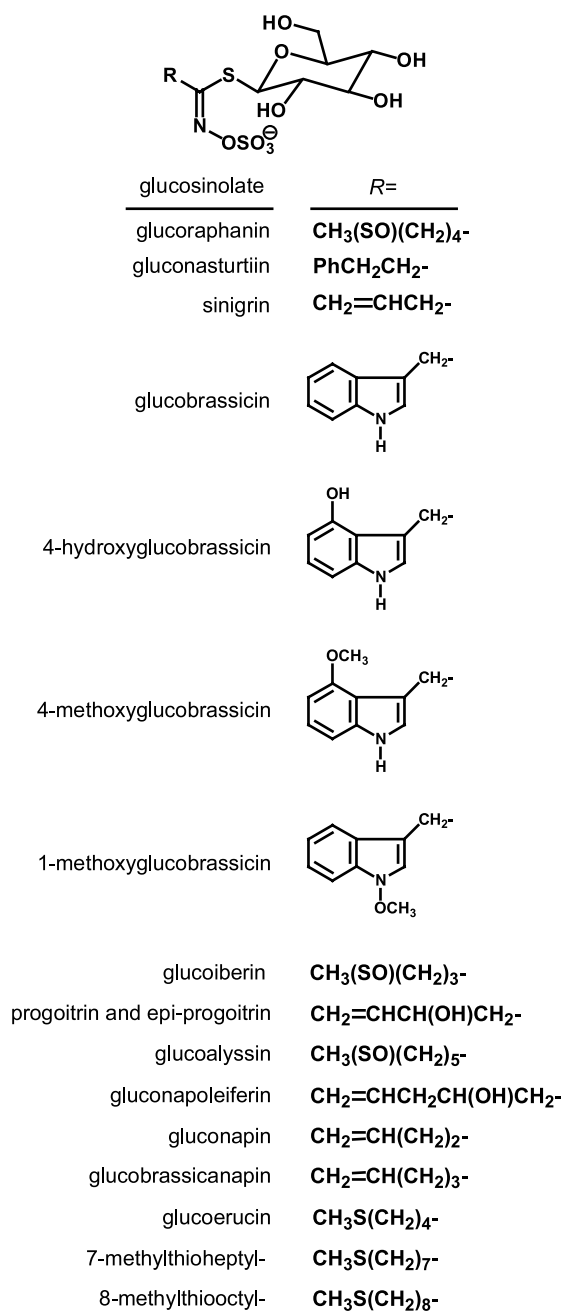
studies, although quantitative analyses of glucosinolates in some of the subspecies included here are apparently not available. Type of cultivar, agronomic and environmental conditions, and postharvest treatments are known to affect glucosinolate levels in plants. One difference between our data and published studies involves watercress. Levels of glucobrassicins in our watercress were somewhat higher than reported elsewhere (55). Our data for pak choi are also somewhat different from published data, as we observed higher levels of 4-methoxyglucobrassicin (49).

The mechanisms by which vegetables protect against lung cancer are poorly understood. For cruciferous vegetables, it appears likely that glucosinolates and their derived ITCs or indole-3-carbinols play an important role as protective agents, because these compounds are unique to this family of vegetables and some efficiently prevent cancer in animal models (23). However, it is well known, and demonstrated again in this study, that there is considerable structural specificity among glucosinolates in different cruciferous vegetables (15-18). It is important to recognize this specificity when formulating

**Table 2. Geometric means (95% confidence intervals) of NNAL, NNAL-Glucs, and total NNAL (pmol/mg creatinine) by levels of smoking intensity and dietary glucobrassicins among 84 Singapore Chinese smokers**

	NNAL	NNAL-Glucs	Total NNAL
No. of cigarettes smoked per day			
≤12 (n = 27)	0.36 (0.21-0.52)	0.72 (0.45-1.02)	1.01 (0.66-1.43)
13-22 (n = 36)	0.50 (0.36-0.65)	1.31 (1.01-1.66)	1.78 (1.36-2.26)
≥23 (n = 21)	0.73 (0.52-0.97)	1.34 (0.94-1.82)	2.02 (1.44-2.74)
P, linear trend	0.01	0.01	0.004
Quartiles of dietary glucobrassicins (μmol/d)			
Q4 (≥105.4)	0.38 (0.21-0.57)	1.07 (0.72-1.49)	1.41 (0.95-1.98)
Q3 (69.8 to <105.4)	0.44 (0.26-0.64)	0.82 (0.51-1.20)	1.21 (0.78-1.74)
Q2 (32.4 to <69.8)	0.61 (0.41-0.83)	1.22 (0.85-1.67)	1.73 (1.21-2.38)
Q1 (<32.4)	0.68 (0.48-0.91)	1.33 (0.93-1.80)	1.97 (1.40-2.68)
P, linear trend	0.01	0.08	0.03

NOTE: Derived from ANOVA with two main effects, cigarettes smoked per day and dietary glucobrassicins (see Subjects and Methods for details).



**Figure 2.** Names and structures of glucosinolates quantified in cruciferous vegetables. The glucosinolate moiety, attached to *R* in the structure at the top, is replaced by  $-\text{N}=\text{C}=\text{S}$  on myrosinase-catalyzed hydrolysis to ITCs (all compounds, except the glucobrassicins) and by  $-\text{OH}$  on hydrolysis of the glucobrassicins.

hypotheses regarding mechanisms of cancer protection by cruciferous vegetables. For example, previous studies have demonstrated that indole-3-carbinol and watercress have opposite effects on levels of urinary total NNAL in smokers (37, 45). Indole-3-carbinol decreases urinary total NNAL presumably by inducing hepatic oxidative

metabolism of NNK, while watercress, probably through release of PEITC, increases levels of urinary total NNAL by blocking oxidative metabolism and/or enhancing glucuronidation (37, 45, 56). In animal models, these mechanisms are protective against lung tumor induction (36, 40, 42-44). In this study, we show that cruciferous vegetable consumption by Chinese smokers in Singapore results in considerable intake of glucobrassicins (up to >42 mg per day). Because glucobrassicins are converted in the body to indole-3-carbinol and its substituted analogues, this provides a reasonable explanation for the observed decreased NNAL in urine. The possible relationship of this phenomenon to lung cancer in these smokers is discussed further below. There was no significant relationship between ITC intake and NNAL levels in this study. Among the ITCs that would be released on vegetable consumption by our subjects, only PEITC, released from gluconasturtiin in watercress, is known to increase levels of total NNAL in urine in animal models (40, 44). Watercress consumption, a good source of PEITC, has been shown to increase total NNAL in smokers (45). However, watercress consumption was modest in our smokers (2.1 g per day), and their average daily dose of gluconasturtiin, the precursor to PEITC, was 2.2  $\mu\text{mol}$  compared with a quartile range of 31 to 104  $\mu\text{mol}$  glucobrassicins. The only other known chemopreventive ITC released in substantial quantities on consumption of these vegetables is sulforaphane (from glucoraphanin in broccoli). Sulforaphane has no effect on NNK-induced lung tumorigenicity in animals and has not been reported to affect NNK metabolism (57). Thus, only by analyzing specific components of cruciferous vegetables were we able to provide a cogent rationale for their effects on NNK metabolism in smokers via released indole-3-carbinols.

In our previous study of the effects of indole-3-carbinol on levels of NNAL and NNAL-Glucs in urine, 13 female smokers were given five consecutive single daily p.o. doses of indole-3-carbinol (400 mg per day), and NNAL and NNAL-Glucs were quantified at baseline and after the 5-day treatment period (37). Significant decreases in urinary levels of NNAL (mean, 23.4%) and NNAL-Glucs (mean, 10.9%) were observed. These results are consistent with those reported here, although the daily dose of indole-3-carbinol was substantially higher in our earlier study (400 vs.  $\geq 15$  mg indole-3-carbinol plus substituted analogues in this study). Also, indole-3-carbinol was given once versus incrementally in the form of vegetables in this study. Indole-3-carbinol is known to induce cytochrome *P*450 1A enzymes probably by binding of its acid decomposition products such as diindolylmethane to the aryl hydrocarbon receptor (17, 58-62). Hepatic *P*450 1A2 is an important catalyst of NNK  $\alpha$ -hydroxylation (63, 64). Therefore, it is completely plausible that exposure to indole-3-carbinols via dietary glucobrassicins, as in our smokers, results in induction of *P*450 1A2 and increased hepatic NNK  $\alpha$ -hydroxylation. In mice treated with indole-3-carbinol, at doses that protect against lung tumorigenesis by NNK, hepatic NNK  $\alpha$ -hydroxylation was induced, resulting in a decreased dose of NNK and NNAL to the lung, less DNA binding in the lung, and less tumor formation (36). In tandem with these changes, lower levels of NNAL and NNAL-Glucs were detected in the urine of NNK/indole-3-carbinol-treated mice than in

control mice treated with NNK only (36). Collectively, these observations are consistent with the results observed here and provide a mechanistic explanation.

However, there are differences between the studies in mice and humans which raise questions about the ultimate benefit of indole-3-carbinols from cruciferous vegetables with respect to protection against lung cancer in smokers. In mice, NNK was administered by i.p. injection. Increased first-pass hepatic clearance of NNK through induction of P450 1A2 resulted in a lower dose of NNK to the lung and protection. However, in smokers, NNK is delivered directly to the lung by inhalation without a first pass through the liver. The effect of indole-3-carbinol on pulmonary enzymes in humans is unknown. It would be expected to induce P450 1A1, but this enzyme is a poor catalyst of NNK metabolism (32). There are reports of P450 1A2 in human lung, and induction of this enzyme by indole-3-carbinol could lead to increased NNK DNA binding in the lung (65). P450 2A13 is probably the best catalyst of NNK  $\alpha$ -hydroxylation in human lung (66). The effects of indole-3-carbinol on this enzyme have not been reported. After uptake of NNK by the lung in smokers, it is partially metabolized to NNAL (32). NNK and NNAL enter the circulation and are metabolized in the liver and elsewhere. Some of the NNAL and NNK is probably returned to the lung periphery via the circulation. (S)-NNAL may bind to receptors there and then be reconverted to NNK (67, 68). Therefore, while indole-3-carbinol may decrease the dose of NNK and NNAL to the lung via induction of hepatic metabolism in smokers, as in mice, it may also induce  $\alpha$ -hydroxylation of NNK or NNAL in the lung, perhaps counteracting this protective effect. These aspects require further study. In particular, it would be important to determine the effects of indole-3-carbinol on inhaled NNK in mice.

In summary, the effects of vegetable consumption on carcinogen metabolism are complex. In this study, we found a correlation between increased levels of glucobrassicins consumed in cruciferous vegetables and decreased amounts of the NNK metabolite NNAL in urine. Previous studies in laboratory animals and humans support the conclusion that this correlation is due to induction of NNK metabolism by indole-3-carbinol and related compounds, which are formed from glucobrassicins. This observation forms the basis for further studies investigating the mechanisms by which vegetable consumption protects against lung cancer.

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## References

- Ziegler RG, Mayne ST, Swanson CA. Nutrition and lung cancer. *Cancer Causes & Control* 1996;7:157-77.
- World Cancer Research Fund/American Institute for Cancer Research. Food, nutrition, and the prevention of cancer: a global perspective. Washington (DC): American Institute for Cancer Research; 1997. p. 143-5.
- Feskanich D, Ziegler RG, Michaud DS, et al. Prospective study of

- fruit and vegetable consumption and risk of lung cancer among men and women. *J Natl Cancer Inst* 2000;92:1812-3.
- Voorrips LE, Goldbohm RA, Verhoeven DT, et al. Vegetable and fruit consumption and lung cancer risk in the Netherlands Cohort Study on diet and cancer. *Cancer Causes & Control* 2000;11:101-15.
- Axelsson G, Rylander R. Diet as risk for lung cancer: a Swedish case-control study. *Nutr Cancer* 2002;44:145-51.
- Wright ME, Mayne ST, Swanson CA, Sinha R, Alavanja MC. Dietary carotenoids, vegetables, and lung cancer risk in women: the Missouri women's health study (United States). *Cancer Causes & Control* 2003;14:85-96.
- Neuhouser ML, Patterson RE, Thornquist MD, Omenn GS, King IB, Goodman GE. Fruits and vegetables are associated with lower lung cancer risk only in the placebo arm of the beta-carotene and retinol efficacy trial (CARET). *Cancer Epidemiol Biomarkers & Prev* 2003;12:350-8.
- Marchand JL, Luce D, Goldberg P, Bugel I, Salomon C, Goldberg M. Dietary factors and the risk of lung cancer in New Caledonia (South Pacific). *Nutr Cancer* 2002;42:18-24.
- Rachtan J. Dietary habits and lung cancer risk among Polish women. *Acta Oncol* 2002;41:389-94.
- Kubik A, Zatloukal P, Tomasek L, et al. Diet and the risk of lung cancer among women. A hospital-based case-control study. *Neoplasma* 2001;48:262-6.
- Alavanja MC, Field RW, Sinha R, et al. Lung cancer risk and red meat consumption among Iowa women. *Lung Cancer* 2001;34:37-46.
- Jansen MC, Bueno-de-Mesquita HB, Rasanen L, et al. Cohort analysis of fruit and vegetable consumption and lung cancer mortality in European men. *Int J Cancer* 2001;92:913-8.
- IARC. IARC handbooks of cancer prevention, vol. 8: fruit and vegetables. Lyon (France): IARC; 2003. p. 323.
- Verhoeven DT, Goldbohm RA, van Poppel G, Verhagen H, Van den Brandt PA. Epidemiological studies of indole glucosinolates and cancer risk. *Cancer Epidemiol Biomarkers & Prev* 1996;5:733-48.
- Fenwick GR, Heaney RK, Mawson R. Glucosinolates. In: Cheeke PR, editor. Toxicants of plant origin, volume II. Glycosides. Boca Raton (FL): CRC Press, Inc.; 1989. p. 2-41.
- Tookey HL, VanEtten CH, Daxenbichler ME. Glucosinolates. In: Liener IE, editor. Toxic constituents of plant stuffs. New York: Academic Press; 1980. p. 103-42.
- McDanell R, McLean AEM, Hanley AB, Heaney RK, Fenwick GR. Chemical and biological properties of indole glucosinolates (glucobrassicins): a review. *Food Chem Toxicol* 1988;26:59-70.
- Fahey JW, Zalcmann AT, Talalay P. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry* 2001;56:5-51.
- Getahun SM, Chung FL. Conversion of glucosinolates to isothiocyanates in humans after ingestion of cooked watercress. *Cancer Epidemiol Biomarkers & Prev* 1999;8:447-51.
- Shapiro TA, Fahey JW, Wade KL, Stephenson KK, Talalay P. Human metabolism and excretion of cancer chemopreventive glucosinolates and isothiocyanates of cruciferous vegetables. *Cancer Epidemiol Biomarkers & Prev* 1998;7:1091-100.
- Hecht SS. Inhibition of carcinogenesis by isothiocyanates. *Drug Metabol Rev* 2000;32:395-411.
- Hecht SS. Chemoprevention by isothiocyanates. In: Kelloff GJ, Hawk ET, Sigman CC, editors. *Cancer chemoprevention volume 1: promising cancer chemopreventive agents*. Totowa (NJ): Humana Press; 2003.
- Hecht SS. Anticarcinogenesis by isothiocyanates, indole-3-carbinol, and allium thiols. Proceedings of the DFG Symposium "Carcinogenic/Anticarcinogenic Factors in Food: Novel Concepts." Wiley-VCH; 1999. p. 306-33.
- Smith TJ, Yang CS. Effect of organosulfur compounds from garlic and cruciferous vegetables on drug metabolism enzymes. *Drug Metabol Drug Interact* 2000;17:23-49.
- Kong AN, Owuor E, Yu R, et al. Induction of xenobiotic enzymes by the map kinase pathway and the antioxidant or electrophile response element (ARE/EpRE). *Drug Metab Rev* 2001;33:255-71.
- Zhang Y, Talalay P. Anticarcinogenic activities of organic isothiocyanates: chemistry and mechanism. *Cancer Res Suppl* 1994;54:1976s-81s.
- London SJ, Yuan JM, Chung FL, et al. Isothiocyanates, glutathione S-transferase M1 and T1 polymorphisms, and lung-cancer risk: a prospective study of men in Shanghai, China. *Lancet* 2000;356:724-9.
- Zhao B, Seow A, Lee EJ, et al. Dietary isothiocyanates, glutathione S-transferase -M1,-T1 polymorphisms and lung cancer risk among Chinese women in Singapore. *Cancer Epidemiol Biomarkers & Prev* 2001;10:1063-7.

29. Spitz MR, Duphorne CM, Detry MA, et al. Dietary intake of isothiocyanates: evidence of a joint effect with glutathione S-transferase polymorphisms in lung cancer risk. *Cancer Epidemiol Biomarkers & Prev* 2000;9:1017-20.
30. Hecht SS. Tobacco smoke carcinogens and lung cancer. *J Natl Cancer Inst* 1999;91:1194-210.
31. Schuller HM. Mechanisms of smoking-related lung and pancreatic adenocarcinoma development. *Nat Rev Cancer* 2002;2:455-63.
32. Hecht SS. Biochemistry, biology, and carcinogenicity of tobacco-specific N-nitrosamines. *Chem Res Toxicol* 1998;11:559-603.
33. Carmella SG, Le Ka KA, Upadhyaya P, Hecht SS. Analysis of N- and O-glucuronides of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNAL) in human urine. *Chem Res Toxicol* 2002;15:545-50.
34. Ren Q, Murphy SE, Zheng Z, Lazarus P. O-Glucuronidation of the lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNAL) by human UDP-glucuronosyltransferases 2B7 and 1A9. *Drug Metab Dispos* 2000;28:1352-60.
35. Hecht SS. Human urinary carcinogen metabolites: biomarkers for investigating tobacco and cancer. *Carcinogenesis* 2002;23:907-22.
36. Morse MA, LaGreca SD, Amin SG, Chung FL. Effects of indole-3-carbinol on lung tumorigenesis and DNA methylation induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and on the metabolism and disposition of NNK in A/J mice. *Cancer Res* 1990;50:2613-7.
37. Taioli E, Garbers S, Bradlow HL, Carmella SG, Akerkar S, Hecht SS. Effects of indole-3-carbinol on the metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in smokers. *Cancer Epidemiol Biomarkers & Prev* 1997;6:517-22.
38. Morse MA, Wang CX, Stoner GD, et al. Inhibition of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced DNA adduct formation and tumorigenicity in lung of F344 rats by dietary phenethyl isothiocyanate. *Cancer Res* 1989;49:549-53.
39. Morse MA, Eklind KI, Amin SG, Hecht SS, Chung FL. Effects of alkyl chain length on the inhibition of NNK-induced lung neoplasia in A/J mice by arylalkyl isothiocyanates. *Carcinogenesis* 1989;10:1757-9.
40. Hecht SS, Trushin N, Rigotty J, et al. Complete inhibition of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone induced rat lung tumorigenesis and favorable modification of biomarkers by phenethyl isothiocyanate. *Cancer Epidemiol Biomarkers & Prev* 1996;5:645-52.
41. Chung FL, Kelloff G, Steele V, et al. Chemopreventive efficacy of arylalkyl isothiocyanates and N-acetylcysteine for lung tumorigenesis in Fischer rats. *Cancer Res* 1996;56:772-8.
42. Staretz ME, Foiles PG, Miglietta LM, Hecht SS. Evidence for an important role of DNA pyridyloxobutylation in rat lung carcinogenesis by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone: effects of dose and phenethyl isothiocyanate. *Cancer Res* 1997;57:259-66.
43. Staretz ME, Koenig L, Hecht SS. Effects of long term phenethyl isothiocyanate treatment on microsomal metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in F344 rats. *Carcinogenesis* 1997;18:1715-22.
44. Boysen G, Kenney PMJ, Upadhyaya P, Wang M, Hecht SS. Effects of benzyl isothiocyanate and 2-phenethyl isothiocyanate on benzo[a]pyrene and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone metabolism in F-344 rats. *Carcinogenesis* 2003;24:517-25.
45. Hecht SS, Chung FL, Richie JP Jr, et al. Effects of watercress consumption on metabolism of a tobacco-specific lung carcinogen in smokers. *Cancer Epidemiol Biomarkers & Prev* 1995;4:877-84.
46. Seow A, Shi CY, Chung FL, et al. Urinary total isothiocyanate (ITC) in a population-based sample of middle-aged and older Chinese in Singapore: relationship with dietary total ITC and glutathione S-transferase M1/T1/P1 genotypes. *Cancer Epidemiol Biomarkers & Prev* 1998;7:775-81.
47. Hankin JH, Stram DO, Arakawa K, et al. Singapore Chinese Health Study: development, validation, and calibration of the quantitative food frequency questionnaire. *Nutr Cancer* 2001;39:187-95.
48. Mac Sharry R. Commission regulation (EEC) no. 1864/90 of 29 June 1990 amending regulation (EEC) no. 1470/68 on the drawing and reduction of samples and on methods of analysis in respect of oil seeds. *Official Journal of the European Communities* 1990;L170:27-34.
49. Lewis J, Fenwick GR. Glucosinolate content of *Brassica* vegetables—Chinese cabbages Pe-tsai (*Brassica pekinensis*) and Pak-choi (*Brassica chinensis*). *J Sci Food Agric* 1988;45:379-86.
50. Carmella SG, Akerkar S, Richie JP Jr, Hecht SS. Intraindividual and interindividual differences in metabolites of the tobacco-specific lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in smokers' urine. *Cancer Epidemiol Biomarkers & Prev* 1995;4:635-42.
51. Snedecor GW, Cochran WG. *Statistical methods*. 6th ed. Ames (IA): Iowa State University Press; 1967.
52. Richie JP, Carmella SG, Muscat JE, Scott DG, Akerkar SA, Hecht SS. Differences in the urinary metabolites of the tobacco-specific lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in black and white smokers. *Cancer Epidemiol Biomarkers & Prev* 1997;6:783-90.
53. Meger M, Meger-Kossien I, Riedel K, Scherer G. Biomonitoring of environmental tobacco smoke (ETS)-related exposure to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). *Biomarkers* 2000;5:33-45.
54. Hecht SS, Murphy SE, Carmella SG, et al. Effects of reduced cigarette smoking on uptake of a tobacco-specific lung carcinogen. *J Natl Cancer Inst* 2004;96:107-15.
55. Rose P, Faulkner K, Williamson G, Mithen R. 7-Methylsulfanylheptyl and 8-methylsulfanyloctyl isothiocyanates from watercress are potent inducers of phase II enzymes. *Carcinogenesis* 2000;21:1983-8.
56. Hecht SS, Carmella SG, Murphy SE. Effects of watercress consumption on urinary metabolites of nicotine in smokers. *Cancer Epidemiol Biomarkers & Prev* 1999;8:907-13.
57. Chung FL, Jiao D, Conaway CC, Smith TJ, Yang CS, Yu MC. Chemopreventive potential of thiol conjugates of isothiocyanates for lung cancer and a urinary biomarker of dietary isothiocyanates. *J Cell Biochem Suppl* 1997;27:76-85.
58. Bradfield CA, Bjeldanes LF. Modification of carcinogen metabolism by indolylic autolysis products of *Brassica oleracea*. In: Friedman M, editor. *Nutritional and toxicological consequences of food processing*. New York: Plenum Press, Inc.; 1991. p. 153-63.
59. Schertzer HG, Sainsbury M. Chemoprotective and hepatic enzyme induction properties of indole and indenoindole antioxidants in rats. *Food Chem Toxicol* 1991;29:391-400.
60. Stresser DM, Williams DE, Griffin DA, Bailey GS. Mechanisms of tumor modulation by indole-3-carbinol. Disposition and excretion in male Fischer 344 rats. *Drug Metabol Dispos* 1995;23:965-75.
61. Kall MA, Vang O, Clausen J. Effects of dietary broccoli on human in vivo drug metabolizing enzymes: evaluation of caffeine, estrone and chlorzoxazone. *Carcinogenesis* 1996;17:793-9.
62. National Cancer Institute. *Clinical development plan: indole-3-carbinol*. *J Cell Biochem* 1996;265:127-36.
63. Smith TJ, Guo Z, Guengerich FP, Yang CS. Metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) by human cytochrome P450 1A2 and its inhibition by phenethyl isothiocyanate. *Carcinogenesis* 1996;17:809-13.
64. Patten CJ, Smith TJ, Murphy SE, et al. Kinetic analysis of the activation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone by heterologously expressed human P450 enzymes and the effect of P450-specific chemical inhibitors on this activation in human liver microsomes. *Arch Biochem Biophys* 1996;332:127-8.
65. Wei C, Cacavale RJ, Kehoe JJ, Thomas PE, Iba MM. CYP1A2 is expressed along with CYP1A1 in the human lung. *Cancer Lett* 2001;164:25-32.
66. Su T, Bao Z, Zhang QY, Smith TJ, Hong JY, Ding X. Human cytochrome P450 CYP2A13: predominant expression in the respiratory tract and its high efficiency metabolic activation of a tobacco-specific carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. *Cancer Res* 2000;60:5074-9.
67. Hecht SS, Carmella SG, Ye M, Le K, Jensen JA, Zimmerman CL, Hatsukami DK. Quantitation of metabolites of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone after cessation of smokeless tobacco use. *Cancer Res* 2002;62:129-34.
68. Wu Z, Upadhyaya P, Carmella SG, Hecht SS, Zimmerman CL. Disposition of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNAL) in bile duct-cannulated rats: stereoselective metabolism and tissue distribution. *Carcinogenesis* 2002;23:171-9.