JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Arecoline *N*-Oxide: Its Mutagenicity and Possible Role as Ultimate Carcinogen in Areca Oral Carcinogenesis

Kuo-Hui Lin,[†] Ching-Yuan Lin,[‡] Chin-Chih Liu,[†] Ming-Young Chou,[§] and Jen-Kun Lin*[,†,§]

[†]Institute of Biochemistry and Molecular Biology and [‡]Department of Otolaryngology, College of Medicine, National Taiwan University, Taipei, Taiwan

[§]College of Oral Medicine, Chung-Shan Medical University, Taichung, Taiwan

ABSTRACT: The areca nut is the most widely consumed psychoactive substance in Taiwan, India, and Southeast Asia. It is considered to be an environmental risk factor for the development of oral submucous fibrosis and cancer. Arecoline, the major alkaloid of areca nut, has been known to cause cytotoxicity and genotoxicity in various systems. However, the active compound accounting for arecoline-induced damage in normal human oral cells is still uncharacterized. The present study was undertaken to identify the active metabolite of arecoline that might induce damage in human oral tissues and cause mutagenicity in *Salmonella typhimurium* tester strains TA 100 and TA 98. It is interesting to find that the major metabolite of arecoline, arecoline *N*-oxide, is moderately mutagenic to these *Salmonella* tester strains. This mutagenicity was potently inhibited by sulfhydryl compounds, namely, glutathione, *N*-acetylcysteine, and cysteine, whereas methionine is inactive in this inhibition. The mutagenicity of arecoline *N*-oxide was strongly inhibited by the *N*-oxide reducing agent titanium trichloride. The possible role of arecoline *N*-oxide in the induction of oral carcinogenesis by areca nut chewing is discussed.

KEYWORDS: areca nut arecoline, arecoline *N*-oxide, mutagenicity, tester strains *Salmonella* TA 98 and TA 100

INTRODUCTION

The alkaloid arecoline is a main constituent of the areca nut, which is chewed by approximately 600 million persons worldwide.[1] There are several different ways in which areca nut is consumed in Taiwan, India, and Southeast Asia, but areca nut chewing is the most common and manifests several pharmacological effects including euphoria, central nervous system stimulation, vertigo, salivation, miosis, tremor, and bradycardia.[2] Epidemiological studies have indicated an association between areca nut chewing and oral precancerous lesions, especially submucous fibrosis and leukoplakia.[3,4] The International Association of Research on Cancer (IARC) has classified the areca nut as a human environmental carcinogen.[2] The habitual chewing of areca nut is proposed to be deleterious to human health, especially in relation to the risk of the development of oral cancer.[2,5] In Taiwan, a group of truck drivers who chewed areca nut heavily and consistently during long-distance driving has a high incidence of oral submucous fibrosis and cancer (C. Y. Lin, personal communication). Several epidemiological efforts have been focused on the interaction of collagen-related genes and susceptibility to betel quid-induced oral submucous fibrosis.[6] A trend was noted for an increased risk of oral submucous fibrosis with increasing number of high-risk alleles for those with both high and low exposures to betel quid or areca nut. The cell selection mechanism of oral fibroblasts is proposed to explain the effect of the modification of cumulative betel quid exposure on the risk profiles of collagen-related genes and phase II enzymes involved in oral carcinogenesis. Case-control studies focused on the interaction between oral cancer risk factors and genetic polymorphisms of cytochrome p450 (CYP)-2E1 and glutathione *S*-transferase (GST) M1 and GSTT1 have been carried out.[7] The results suggested that there are gene−gene and gene−environment interactions in the development of oral cancer.

The most important risk factors for oral cancer in Taiwan are cigarette smoking, alcohol drinking, and betel quid (areca nut) chewing.[8] The environment−gene interaction on carcinogenesis has been well demonstrated by phase I and II enzymes that are involved in the metabolism of carcinogens. Some of these enzymes are polymorphic in genotypes, with corresponding variation in their activities. Many procarcinogenic compounds remain inactive until they are enzymatically converted to an electrophilic species that is capable of covalently binding to DNA and leading to mutation.[8] A lot of xenobiotics continue to be found that are metabolized in mammals to electrophilic reactants that form covalently bound adducts to cellular DNA, leading to tumor formation.[9] Thus, metabolic activation is considered to be a critical step in mutation and carcinogenesis.[10,11] The *Salmonella typhimurium* mutagenicity test (commonly called the Ames test) is used worldwide as an initial screening to determine the mutagenic potential of new chemicals for hazard identification.[12] S9 fractions prepared from the livers of rats pretreated with phenobarbital/5,6-benzoflavone or Aroclor 1254 to induce drug-metabolizing enzyme activity are very useful for mutagenicity screening systems, because they effectively metabolically activate promutagens to mutagen and are convenient to operate.[13]

On the basis of the intensive epidemiological investigations, the strong association of areca nut chewing with oral cancer in Taiwan has been indicated. The major task ahead is to identify the active carcinogen in the areca nut causing the oral submucosal fibrosis and cancer. Despite long-term efforts, the role of areca alkaloids such as arecoline in the adverse health effects of areca

Received: December 15, 2010
Revised: February 13, 2011
Accepted: February 14, 2011
Published: March 03, 2011

nut chewing remains to be elucidated. In this study, we have focused our efforts on the identification of naturally occurring mutagens or promutagens in the areca nut. The principal metabolite of arecoline is arecoline *N*-oxide, the metabolism of which has been recently studied. Recent studies by the Gonzalez group[14] have demonstrated that the principal pathways of the metabolism of arecoline *N*-oxide (also called arecoline 1-oxide) were mercapturic acid formation, with catabolism to mercaptan and methyl-mercaptan metabolites, apparent C=C double bond reduction, carboxylic acid reduction to the aldehyde, *N*-oxide reduction, and de-esterification. The mutagenicity and carcinogenicity of these metabolites have never been investigated.

Here, we report our investigation on the mutagenicity of arecoline and arecoline *N*-oxide in *Salmonella* tester strains TA 100 and TA 98.

MATERIALS AND METHODS

Chemicals. Arecoline hydrobromide, histidine, D-biotin, $NADP^+$, glucose-6-phosphate, 4-nitroquinoline-*N*-oxide (4NQO), benzo(*a*)pyrene (BaP), and Aroclor 1254 (polychlorinated biphenyl, PCB) were purchased from Sigma (St. Louis, MO). Titanium trichloride ($TiCl_3$) was purchased from Sigma-Aldrich Laborchemikallen GmbH (Seelze, Germany). Arecoline *N*-oxide (also called arecoline 1-oxide) was synthesized from arecoline hydrobromide and peracetic acid as previously described.[14,15] The synthesized product has been confirmed by mass spectrometry to give the molecular ion mass (m/z) of 171.7 and by NMR analysis (see Figure 7). The synthesized arecoline *N*-oxide was reduced by $TiCl_3$ to arecoline as checked by thin layer chromatography.

Bacterial Strains. A set of histidine-requiring strains was used for mutagenicity test. The tester strains used for the Ames test were *Salmonella typhimurium* TA 100 and TA 98. The TA 100 strain was used to detect base pair substitution mutations, whereas TA 98 was used to detect frame-shift mutation, and these two strains were developed by Prof. B. N. Ames of the University of California, Berkeley, CA.[12] The subcultures of *S. typhimurium* TA 100 and TA 98 were kindly provided by Dr. Fu-Chu Peng, Institute of Toxicology, College of Medicine, National Taiwan University, Taipei, Taiwan.

Preparation of Rat Liver S9 Fraction [16]**.** The S9 fractions used in this study was prepared from the liver of male Sprague−Dawley rats administered intraperitoneal injection of the inducer Aroclor 1254 (PCB) in corn oil (500 mg/kg). Five days later, the rats were sacrificed, the livers were removed aseptically, and the S9 fractions were prepared according to the procedure of Garner et al.[16] S9 mix comprised the S9 fraction and cofactors including glucose 6-phsphate, $NADP^+$, and glucose 6-phosphate dehydrogenase. The requirement of these cofactors has been described by Garner et al.[17] and by Maron and Ames.[12]

Top Agar and Agar Plate. Top agar containing 0.6% Difco agar and 0.5% NaCl was autoclaved and stored at room temperature in a volume of 100 mL. The agar plate for the mutagenicity assay contained 30 mL of minimal glucose agar medium. The medium was made from 1.5% Bacto-Difco agar and 2% glucose in Vogel-Bonner medium E.[18]

Mutagenicity Assay. The Ames test (preincubation method) at 37 °C for 30 min was conducted to examine the mutagenicity of the tested chemicals.[12,17] The procedure was performed as described by Maron and Ames.[12] Both BaP and 4NQO were used as positive controls in these tests. A volume of 0.5 mL of S9 mix in 0.1 M phosphate buffer, pH 7.4, was added to a sterile (13 × 100 mm) capped culture tube placed in an ice bath; 0.1 mL of test compound solution was added and mixed. Then 0.1 mL of bacterial culture was added, and the mixture was vortexed gently and incubated at 37 °C for 30 min. Finally, 2 mL of top agar was added and mixed by vortexing the soft agar for 3 s at low speed and then poured onto a minimal glucose agar plate. To achieve a uniform distribution of the top agar on the surface of the plate, it was necessary to tilt and rotate the uncovered plate and then place it, covered, on a level surface to harden. The plates were covered promptly with brown paper to avoid the effects of light on photosensitive chemicals. Within an hour the plates were inverted (to avoid the condensed water drop from damaging the growing bacterial colonies on the agar surface) and placed in a dark, vented, 37 °C incubator. After 48 h, the revertant colonies on the test plates and on the control plates were counted.

Table 1. Mutagenicity of Arecoline in *Salmonella typhimurium* TA 100 (with or without S9)

	revertants[a] (mean ± SD)	
assay system	− S9	+ S9
0.1 M phosphate buffer, pH 7.4	102 ± 6	110 ± 34
BaP (5 μg/plate)	105 ± 7	530 ± 98
4NQO (0.05 μg/plate)	624 ± 27	
arecoline (1 μg/plate)	103 ± 5	210 ± 11
arecoline (10 μg/plate)	116 ± 5	190 ± 28
arecoline (50 μg/plate)	110 ± 2	192 ± 39
arecoline (100 μg/plate)	115 ± 3	145 ± 27
arecoline (200 μg/plate)	113 ± 2	142 ± 18

[a] Data from the average of three determinations (mean ± SD, SD for standard deviation). S9, rodent microsome preparation, see Materials and Methods for details.

Table 2. Mutagenicity of Arecoline in *Salmonella typhimurium* TA 98 (with or without S9)

	revertants[a] (mean ± SD)	
assay system	− S9	+ S9
0.1 M phosphate buffer, pH 7.4	10 ± 1	16 ± 2
BaP (5 μg/plate)		702 ± 186
4NQO (0.05 μg/plate)	58 ± 6	
arecoline (1 μg/plate)	11 ± 2	25 ± 6
arecoline (10 μg/plate)	11 ± 1	17 ± 3
arecoline (50 μg/plate)	10 ± 1	20 ± 4
arecoline (100 μg/plate)	13 ± 3	15 ± 3
arecoline (200 μg/plate)	13 ± 2	18 ± 7

[a] Data from the average of three determinations (mean ± SD, SD for standard deviation). S9, rodent microsome preparation, see Materials and Methods for details.

RESULTS

Mutagenic Effect of Arecoline in the Presence of S9. The mutagenic effect of the major alkaloid of areca nut, arecoline, was tested in *S. typhimurium* TA 100 and TA 98 in the absence or presence of rat liver S9 preparation. The results are summarized in Tables 1 and 2, respectively. As indicated in Table 1, no significant mutagenic effects of arecoline were detected in *S. typhimurium* TA 100 in the absence of S9, whereas a low mutagenic effect of this compound was detected in the presence of S9. A similar trend was observed in *S. typhimurium* TA 98 (Table 2). Both BaP and 4NQO were used as positive controls in these tests.

Mutagenic Effect of Arecoline *N*-Oxide. The mutagenic effect of arecoline *N*-oxide was tested in *S. typhimurium* TA 100 and TA 98 in the absence of S9. The results are summarized in Table 3. It is interesting to find that a dose-dependent

Table 3. Mutagenicity of Arecoline *N*-Oxide in *Salmonella typhimurium* TA 100 and TA 98

	revertants[a] (mean ± SD)	
assay system	TA 100	TA 98
0.1 M phosphate buffer, pH 7.4	101 ± 17	10 ± 2
4NQO (0.05 μg/plate)	636 ± 11	68 ± 14
arecoline *N*-oxide (1 μg/plate)	139 ± 7	17 ± 5
arecoline *N*-oxide (5 μg/plate)	142 ± 14	22 ± 3
arecoline *N*-oxide (10 μg/plate)	163 ± 28	33 ± 3
arecoline *N*-oxide (50 μg/plate)	228 ± 21	35 ± 2
arecoline *N*-oxide (100 μg/plate)	307 ± 29	42 ± 4
arecoline *N*-oxide (200 μg/plate)	395 ± 9	44 ± 2
arecoline *N*-oxide (500 μg/plate)	604 ± 7	54 ± 3
arecoline *N*-oxide (1000 μg/plate)	964 ± 50	86 ± 5

[a] Data from the average of three determinations (mean ± SD), SD for standard deviation.

mutagenic effect of arecoline *N*-oxide in both tester strains TA 100 and TA 98 was observed. The dose ranges of the compound were from 0.5 to 500 μM/plate, and the numbers of revertants produced were from 139 to 964 (for TA 100) and from 17 to 86 (for TA 98), respectively (Figure 1). The negative control system (0.1 M phosphate buffer, pH 7.4) gave the background revertants 102 (for TA 100) and 10 (for TA 98), respectively. The positive control 4NQO (0.05 μM/plate) gave the revertants 636 (for TA 100) and 68 (for TA 98), respectively. Here, the net revertants number of 4NQO for TA 100 after correction to the background values is 534 (636−102). This value is comparable to a recent collaborative study paper by Hakura et al.,[19] which gave 5400 revertants per micromolar of 4NQO per plate. It is expected in their experimental conditions, 0.05 μM 4NQO could give 270 revertants. Many factors could affect the mutagenicity of a testing compound; it is considered that the value 270 revertants obtained by Hakura et al.[19] is comparable to our value of 534 revertants.

Inhibitory Effects of Sulfhydryl Compounds on the Mutagenicity of Arecoline *N*-Oxide. Several papers have indicated that glutathione (GSH) and sulfhydryl compounds could suppress the toxicity and carcinogenicity of areca nut extracts.[20−23] The effects of several sulfhydryl compounds including GSH, *N*-acetylcysteine (NAC), and cysteine on the mutagenicity of arecoline *N*-oxide in *S. typhimurium* TA 100 and TA 98 were investigated. The results are summarized in Figure 2 and Tables 4 and 5. All sulfhydryl compounds were very active in inhibiting the mutagenicity of arecoline *N*-oxide. On the basis of the data presented in Figure 2A, arecoline *N*-oxide (5 μmol/plate) could produce 842 and 62 revertants for TA 100 and TA98, respectively. GSH at 50 nmol/plate could reduce the revertants to 440 and 29, respectively. When the concentration of GSH was increased to 500 nmol/plate, the mutagenicity of arecoline *N*-oxide was completely inhibited. A very similar trend was observed in the case of NAC (Figure 2A). It is interesting to note that $^1/_{10}$ molar concentration of GSH (500 nmol/plate) or NAC (500 nmol/plate) is enough to inhibit completely the mutagenicity of arecoline *N*-oxide (5000 nmol or 5 μmol/plate).

Another sulfhydryl amino acid, cysteine, also showed a similar inhibitory effect on the mutagenicity of arecoline *N*-oxide (Figure 2B). On the contrary, another sulfur-containing amino acid, methionine (up to 5000 nmol/plate), was completely inactive in this test (Figure 2B).

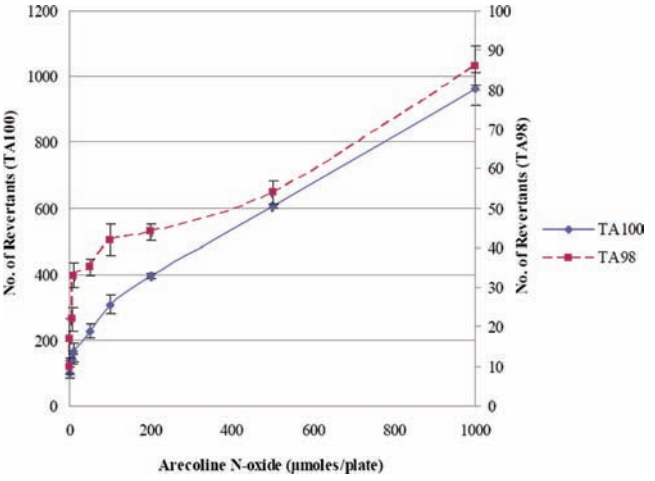

Figure 1. Mutagenicity of arecoline *N*-oxide in the *Salmonella* tester strains TA 100 and TA 98. The mutagenicity of arecoline *N*-oxide was assayed as described under Materials and Methods. *X*-axis: concentration of arecoline *N*-oxide in μmol/plate; *Y*-axis: number of revertants TA 100 in black and those of TA 98 in red.

Inhibitory Effect of Titanium Trichloride ($TiCl_3$) on the Mutagenicity of Arecoline *N*-Oxide. $TiCl_3$ is a specific reducing agent for *N*-oxide.[24] The effect of $TiCl_3$ on the mutagenicity of arecoline *N*-oxide was studied, and the results are given in Table 3 and shown in Figure 3. Extremely potent inhibition was detected at a concentration as low as 0.007 nmol/plate. In this test the mutagenicity of arecoline *N*-oxide (5.8 μmol/plate) produced 856 (TA 100) and 68 (TA 98) revertants. The mutagenicity was completely inhibited by 70 nmol/plate of $TiCl_3$ (Table 3). Attenuation of the mutagenicity of arecoline *N*-oxide and 4NQO has been observed through metabolic inactivation or passive adsorption of the compound on S9 (Figures 4). These findings suggested that the *N*-oxide group in arecoline *N*-oxide is essential for its mutagenicity. Furthermore, the inactivation of mutagenicity might be due to either enzyme action or physical adsorption of S9.

Rat liver S9 preparation contains a lot of metabolic activation enzymes for xenobiotics; it also contains several metabolic inactivation enzymes.[10−14] In this study, we have found that the rat liver S9 preparation could attenuate the mutagenicities of arecoline *N*-oxide and 4NQO through metabolic inactivation (Figure 4A) or possible physical adsorption (Figure 4B). The effects of pH on the mutagenicity of arecoline *N*-oxide have been studied (Figure 5). It seemed that arecoline *N*-oxide is heat-laibile and pH-sensitive; therefore, the effects of various pH values on the mutagenicity of arecoline *N*-oxide in *S. typhimurium* TA 100 and TA 98 were studied, and the results are illustrated in Figure 6. Both tester strains showed maximum mutagenicity at pH 6.0 and a moderate mutagenicity plateau between pH 7.0 and 10. Both strains showed low mutagenicity at acidic pH (2.0) and alkaline pH (12.0).

Effect of Temperature on the Mutagenicity of Arecoline *N*-Oxide. The effects of temperature on the mutagenicity of arecoline *N*-oxide were investigated, and the results are illustrated in Table 6. A solution of arecoline *N*-oxide (1000 μg/100 μL) in 0.1 M phosphate buffer, pH 7.4, was incubated at different temperatures (25, 35, 45, 55, 65, 75, 85, and 100 °C) for 30 min. The incubated arecoline *N*-oxide solutions were used for mutagenicity assay. The results are illustrated in Figure 6. It appeared

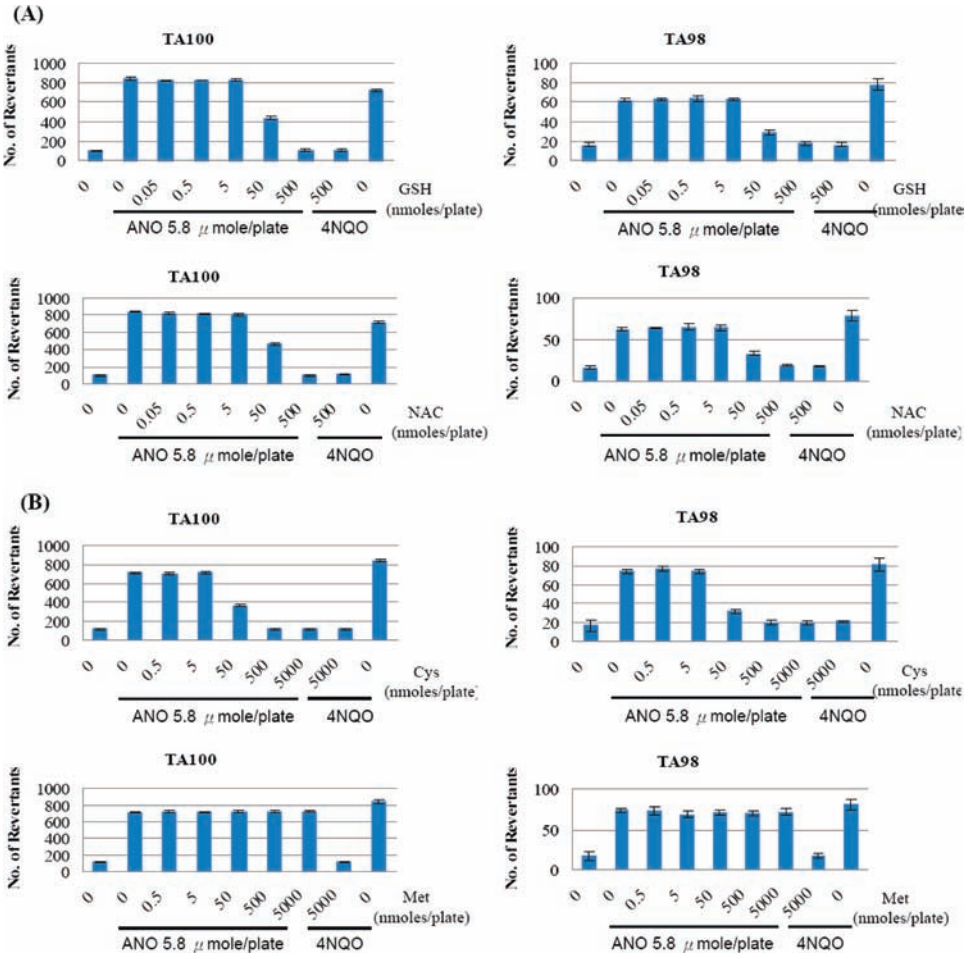

Figure 2. (A) Effects of glutathione (GSH) and *N*-acetylcysteine (NAC) on the muatgenicity of arecoline *N*-oxide. The sulfhydryl compounds (GSH or NAC) were mixed with tester bacterial cells before arecoline *N*-oxide. *X*-axis: concentrations of sulfhydryl compounds (various concentrations, μmol/plate) and arecoline *N*-oxide 5 μmol/plate and 4NQO 0.05 μmol/plate. *Y*-axis: number of revertants. (B) Effects of cysteine and methionine on the mutagenicity of arecoline *N*-oxide. The experimental conditions were as described for panel A.

that at a temperature of >45 °C arecoline *N*-oxide could lose mutagenicity remarkably. The compound was found to be stable at −20 to −70 °C on storage.

DISCUSSION

Effect of S9 Mix on the Mutagenicity of Areca Nut and Arecoline. The present study has demonstrated that the major areca nut alkaloid arecoline is nonmutagenic as such (without S9) to *S. typhimurium* TA 100 and TA 98, but it will become weakly mutagenic after rat liver S9 treatment (Table 3). The active mutagenic compound that generated in the S9-treated system is unknown, but it could be possibly one of its metabolites. On the basis of previous studies,[14,25] the principal metabolite of arecoline is arecoline *N*-oxide (arecoline-1-oxide), the metabolism of which has been studied.[14]

The main pathways of metabolism of arecoline *N*-oxide were mercapturic acid formation, *N*-oxide reduction, and de-esterification. Approximately 50% of the urinary metabolites corresponded to unchanged arecoline *N*-oxide. Most of these metabolites are formed during its catabolic detoxification reactions.[14] It is concluded that arecoline *N*-oxide is the most prominent and important metabolite of arecoline in the studied animals. For this reason, arecoline *N*-oxide was selected as the main target of this mutagenicity test. It is encouraging to see that arecoline *N*-oxide is potently mutagenic to both *Salmonella* tester strains TA 100 and TA 98 (Table 3). The effect of S9 mix on the mutagenicity of areca nut and arecoline on areca nut chewers to develop oral carcinogenesis,[3,4,6,8] which comprised initiation, promotion and progression step.[8,9] A rather complicated step was found to be involved in the initiation of areca nut carcinogenesis. This step included metabolic activation of arecoline to arecoline *N*-oxide by a microsomal cytochrome P450[14] system and by flavin-containing monooxygenase.[25] Meanwhile, the arecoline *N*-oxide would be deoxygenated by microsomal cytochrome P450 system[14] and reduced by sulfhydryl compounds such as GSH and SH-containing proteins. The final available levels of arecoline *N*-oxide will control the initiation of areca carcinogenesis.[1]

It has been demonstrated in the present study that arecoline and its metabolite arecoline *N*-oxide might play important roles in the oral toxicity and oral carcinogeneicity of areca-chewing peoples. The attenuation of arecoline-*N*oxide mutagenicity by S9 may decrease the oral toxicity and carcinogenesis in the target tissues, and this suppression mechanism may reflect the low carcinogenicity of areca nut chewing in the population clinically. A person must chew areca nut for a long time to develop oral carcinogenesis.[3]

Table 4. Effects of Reduced Glutathione (GSH) and *N*-Acetylcysteine (NAC) on the Mutagenicity of Arecoline *N*-Oxide in *Salmonella typhimurium* TA 100 and TA 98

	revertants[a] (mean ± SD)	
assay system	TA 100	TA 98
0.1 M phosphate buffer, pH 7.4	103 ± 8	16 ± 2
4NQO (0.05 μg/plate)	720 ± 16	78 ± 6
arecoline *N*-oxide (5 μg/plate)	842 ± 10	62 ± 2
+ GSH (0.05 nmol/plate)	825 ± 8	63 ± 1
+ GSH (0.5 nmol/plate)	825 ± 3	64 ± 3
+ GSH (5 nmol/plate)	826 ± 10	63 ± 1
+ GSH (50 nmol/plate)	440 ± 15	29 ± 3
+ GSH (500 nmol/plate)	105 ± 8	18 ± 2
+ NAC (0.05 nmol/plate)	823 ± 10	64 ± 1
+ NAC (0.5 nmol/plate)	815 ± 11	65 ± 4
+ NAC (5 nmol/plate)	806 ± 9	64 ± 4
+ NAC (50 nmol/plate)	459 ± 12	33 ± 2
+ NAC (500 nmol/plate)	103 ± 7	19 ± 1
GSH alone (500 nmol/plate)	105 ± 14	16 ± 2
NAC alone (500 nmol/plate)	114 ± 8	18 ± 1

[a] Data from the average of three determinations (mean ± SD), SD for standard deviation.

Table 5. Effects of Cysteine and Methionine on the Mutagenicity of Arecoline *N*-Oxide in *Salmonella typhimurium* TA 100 and TA 98

	revertants[a] (mean ± SD)	
assay system	TA 100	TA 98
0.1 M phosphate buffer, pH 7.4	119 ± 7	17 ± 6
4NQO (0.05 μg/plate)	840 ± 17	81 ± 7
arecoline *N*-oxide (5 μg/plate)	712 ± 6	74 ± 2
+ cysteine (0.5 nmol/plate)	699 ± 12	77 ± 3
+ cysteine (5 nmol/plate)	715 ± 9	74 ± 2
+ cysteine (50 nmol/plate)	370 ± 14	32 ± 2
+ cysteine (500 nmol/plate)	118 ± 6	20 ± 3
+ cysteine (5000 nmol/plate)	117 ± 3	19 ± 2
+ methionine (0.5 nmol/plate)	729 ± 14	73 ± 4
+ methionine (5 nmol/plate)	718 ± 12	69 ± 4
+ methionine (50 nmol/plate)	727 ± 12	71 ± 3
+ methionine (500 nmol/plate)	723 ± 20	70 ± 3
+ methionine (5000 nmol/plate)	724 ± 8	72 ± 4
cysteine alone (5000 nmol/plate)	120 ± 6	21 ± 2
methionine alone (5000 nmol/plate)	117 ± 3	17 ± 3

[a] Data from the average of three determinations (mean ± SD), SD for standard deviation.

Effect of Sulfhydryl Compounds on the Mutagenicity of Arecoline *N*-Oxide. The mutagenicity of arecoline *N*-oxide was strongly inhibited by sulfhydryl compounds such as GSH, NAC, and cysteine (Figure 2). The mechanism of this inhibition is not well-elucidated. It might be due to the molecular interaction of arecoline *N*-oxide with sulfhydryl compounds to form adducts or the reducing ability of −SH groups, which may remove *N*-oxide by reduction.

Effect of Titanium Trichloride on the Mutagenicity of Arecoline *N*-Oxide. The mutagenicity of arecoline *N*-oxide was selectively inhibited by titanium trichloride (Figure 3). $TiCl_3$ (700 nmol/plate) could completely block the mutagenicity of arecoline *N*-oxide (5.8 μmol or 5800 nmol/plate). It is obvious that the complete inhibition was achieved at the molar ratio of 700/5800 = 1/8. $TiCl_3$ is a well-known specific reducing agent for *N*-oxide and nitrate.[26] The mechanism of this inhibition could be due to its *N*-oxide reduction but needed further investigation was found in several SH-proteins in the cells. In the present study, these sulfhydryl compounds were found to effectively inhibit the mutagenecity of arecoline *N*-oxide (Figure 2); therefore, these sulfhydryl compouns could act as inhibitors of areca nut carcinogenesis. Several observations[20,21,23] confirmed these protective effects. Antioxidants such as GSH and NAC can potentially prevent such arecoline cytotoxicity, and carcinogenesis has been demonstrated by Jeng et al.[20] Previous studies indiated that Taiwan areca nut is a promoter rather than an initiator during the carcinogenesis of hamster bucal pouch carcinoma induction.[21] GSH was shown to inhibit the promotion of this experiment. Another study showed that hamsters chewing betel quid or areca nut show epithelial hyperplasia of the cheek pouch; meanwhile, GSH significantly inhibits this pathological change.[23] Furthermore, addition of GSH reduced the cytotoxic and morphological alterations of the transformed fibroblasts treated with aqueous extracts of areca nut.[22] The present study was undertaken to evaluate the effect of glutathione on areca nut treated normal human bucal fibroblast culture and its potential as a chemopreventive agent.[23]

Comparison of Arecoline *N*-Oxide and 4NQO on Oral Carcinogensis. Chemically, both arecoline *N*-oxide and 4NQO are classified in the *N*-oxides category. The mutagenicities of these two compounds are inhibited by incubation with rat liver S9 preparation (Figures 4). Both 4NQO and 1-nitropyrene are well-known mutagenic and carcinogenic aromatic nitro compounds. Their mutagenicities were suppressed by the presence of rat liver S9.[26] A reduction in the amount of pro-mutagen through their microsomal detoxification, such as for hydroxylated metabolites,[27] is one possible reason for the decrease in mutagenicity seen in the presence of rat liver S9. In the case of arecoline *N*-oxide, metabolic *N*-oxide reduction[14] may play a substantial role in the suppression of mutagenicity. The present data indicating the strong inhibitory effects of $TiCl_3$ on the mutagenicity of arecoline *N*-oxide (Figure 3) have supported this contention.

In a comparison of the potencies of mutagenicities of 4NQO and arecoline *N*-oxide (Table 3), the mutagenicity of 4NQO is approximately 10000-fold higher than that of arecoline *N*-oxide. However, arecoline *N*-oxide should be considered as an active mutagen in the present test system. It is worth pointing out that the mutagenicity of a compound may vary with the different species of organisms. Furthermore, the mutagenicity and carcinogenicity of a tested compound are related but not always parallel.[8,11]

Clinical observation in head−neck surgeries have convincingly revealed that in Taiwan most oral cancer patients admitted to the hospital have chewed betel quid or areca nut for more than 10 years (C. Y. Lin et al., unpublished data). Furthermore, in India and China, most cases of oral submucosal fibrosis were found in chewers who had chewed betel quid for a long period of time.[3,24] These observations strongly suggested that the mutagenic and carcinogenic substance(s) encountered in the processes of areca chewing might be rather weak but consistent-acting mutagens or carcinogens. It seemed that arecoline or it metabolite arecoline *N*-oxide might be a promising candidate for involvement in inducing oral carcinogenesis.

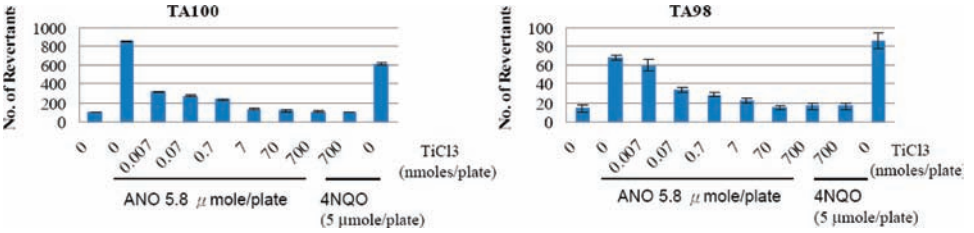

Figure 3. Effects of titanium trichloride on the mutagenicity of arecoline *N*-oxide. *X*-axis: concentration of titanium trichloride (in various concentrations, nmol/plate) plus arecoline *N*-oxide 5.8 μmol/plate) or 4NQO, 5.8 μmol/plate. *Y*-axis: number of revertants.

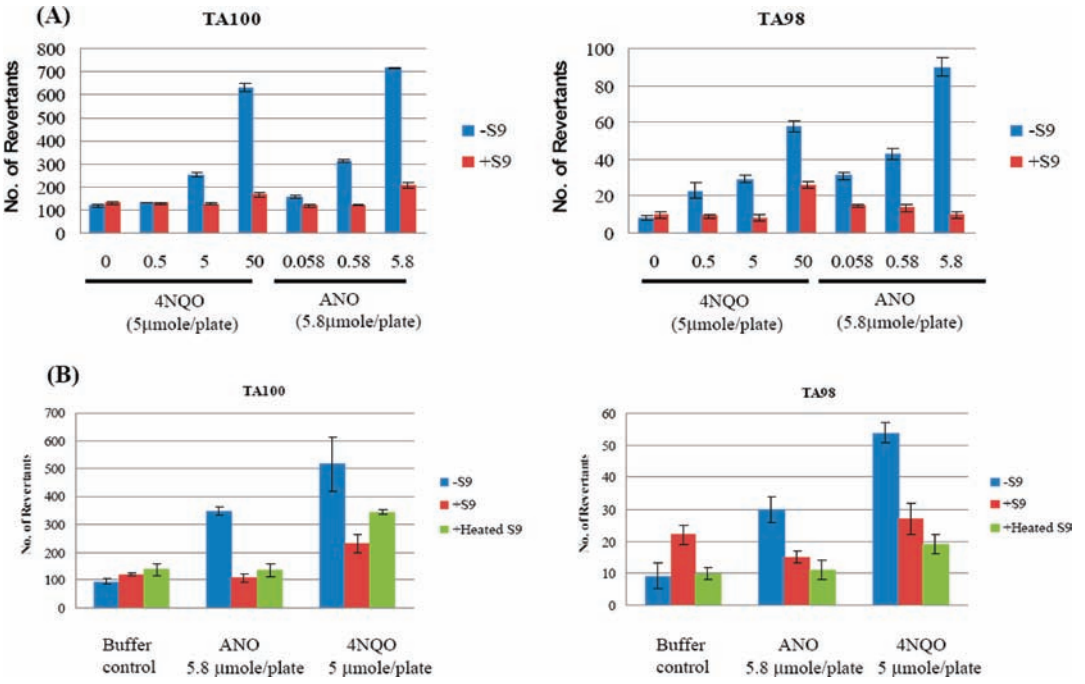

Figure 4. (A) Effects of freshly prepared S9 mix on the mutagenicity of arecoline *N*-oxide and 4NQO. The experimental conditions were as described for Figure 2A and under Materials and Methods. *X*-axis: concentrations of arecoline *N*-oxide (ANO), 5.8 μmol/plate; 4NQO, 5.8 μmol/plate. *Y*-axis: average number of revertants (from three determinations) in the absence of S9 mix (blue bar) or in the presene of S9 mix (red bar). (B) Effects of heated S9 mix on the mutagenicity of ANO and 4NQO.

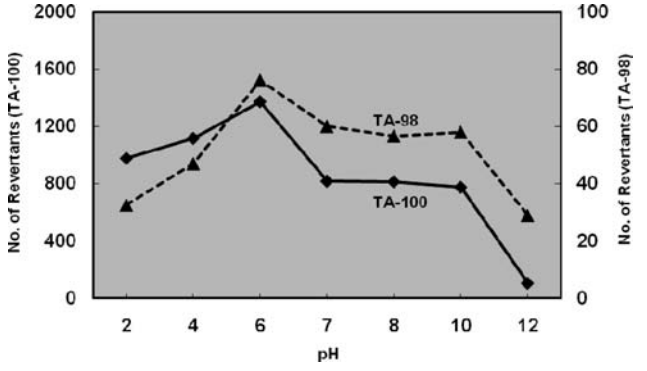

Figure 5. Effects of various pH values on the mutagenicity of arecoline *N*-oxide. The mutagenicity of arecoline *N*-oxide was assayed at various pH values of the prepared buffer solutions including phosphate−citrate buffer (pH 2.6−7.0), phosphate buffer (pH 5.7−8.0), and carbonate−bicarbonate buffer (pH 9.2−10.7). The preparation of these buffer solutions was described by Gomori.[28] The revertants of TA 100 are indicated by a solid line, whereas those of TA 98 are indicated by a broken line.

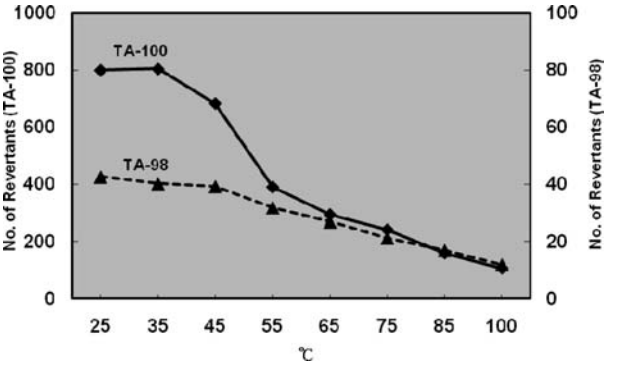

Figure 6. Effect of different temperatures on the mutagenicity of arecoline *N*-oxide. A solution of arecoline *N*-oxide (1000 μg/100 μL) in 0.1 M phosphate buffer, pH 7.4, was incubated at different temperatures (25−100 °C) for 30 min. The mutagenicity of the resulting solutions was assayed as described under Materials and Methods. The revertant changes of TA 100 are indicated by a solid line, whereas those of TA 98 are indicated by a broken line.

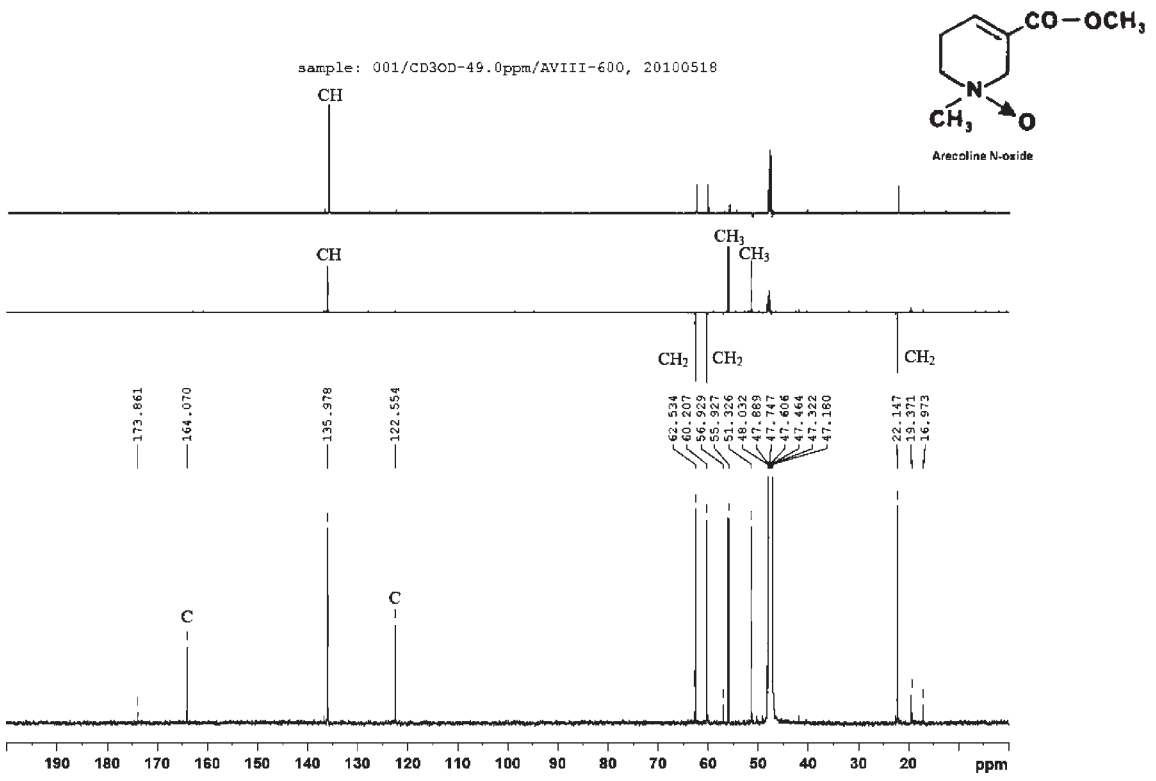

Figure 7. NMR spectrum of arecoline *N*-oxide. This compound was subjected to ^{13}C NMR (Bruker Cyro AV 600 NMR) analysis, which confirmed the identity of arecoline *N*-oxide: ^{13}C NMR (600 MHz, methanol-d_3) δ 164.070, 135.978, 122.554, 62.534, 60.207, 55.927, 51.326, 22.147.

Table 6. Effects of $TiCl_3$[a] on the Mutagenicity of Arecoline *N*-Oxide in *Salmonella typhimurium* TA 100 and TA 98

	revertants[b] (mean ± SD)	
assay system	TA 100	TA 98
0.1 M phosphate buffer, pH 7.4	98 ± 4	14 ± 4
4NQO (0.05 μg/plate)	609 ± 16	86 ± 8
arecoline *N*-oxide (5.8 μg/plate)	856 ± 8	68 ± 3
+ $TiCl_3$ (0.007 nmol/plate)	316 ± 8	60 ± 6
+ $TiCl_3$ (0.07 nmol/plate)	269 ± 6	33 ± 3
+ $TiCl_3$ (0.7 nmol/plate)	232 ± 8	28 ± 2
+ $TiCl_3$ (7 nmol/plate)	131 ± 4	22 ± 3
+ $TiCl_3$ (70 nmol/plate)	108 ± 11	15 ± 2
+ $TiCl_3$ (700 nmol/plate)	102 ± 5	14 ± 2
$TiCl_3$ alone (700 nmol/plate)	96 ± 4	16 ± 3

[a] $TiCl_3$, titanium trichloride, a specific reducing agent for *N*-oxide. [b] Data from the average of three determinations (mean ± SD), SD for standard deviation.

Arecoline is the main water-soluble alkaloid of areca nut. During areca nut chewing, the concentration of arecoline was reported to reach around 140 μg/mL in saliva;[28] it is absorbed in the buccal cavity and be detected in fasting blood in a dose- and time-dependent manner.[29] After efficient quantitative metabolic conversion, 140 μg/mL of arecoline might be theoretically converted to 170 μg/mL of arecoline *N*-oxide, which might give approximately 307 revertants (or higher) of TA 100 and 42 revertants (or higher) of TA 98 under the present mutagenicity assay conditions (the revertant values were extrapolated from the values in Figure 2A, see further discussion below).

On the basis of the data in Figure 2A, arecoline *N*-oxide at 100 μg/plate could give 307 revertants for TA 100 and 42 revertants for TA 98. The mutagenicity assays were carried out in a preincubation mixture of 0.7 mL. Therefore, the final concentration of arecoline *N*-oxide in reaction mixture is 100 μg/0.7 mL = 142 μg/mL. As mentioned above, we have demonstrated the mutagenicity of arecoline *N*-oxide in *S. typhimurium* TA 100 and TA 98. It seemed that the effective concentration of this *N*-oxide was 1 μg/plate.[30] On the basis of the multistep processes,[29,31] the concentration of arecoline in the saliva or blood of areca nut chewers could reach 140 μg/mL (this is equivalent to 170 μg/mL of arecoline *N*-oxide; apparently, this value is higher than 142 μg/mL). In conclusion, in the present study carcinogenesis is characterized by initiation, promotion, and progression.

Relationship between Arecoline *N*-Oxide and Oral Carcinogenesis Based on Clinical Observation. The most used animal models for oral cancer research are the hamster bucal pouch by fat-soluble DMBA and rat tongue tumor by water-soluble 4NQO and carcinogen.[30] The multistep processes of carcinogenesis are characterized by initiation, promotion, and progression. Chronic administration of 4NQO in drinking water stimulates rat tongue carcinogenesis like human 7,12-dimethylbenzanthracene (DMBA) and rat tongue by water-soluble 4NQO. One of the most important routes of oral carcinogen is through liquid containing a water-soluble carcinogen. 4NQO is well suited to examine the role of xenobiotics in experimental oral carigenesis.[30,32,33] High similarity was found between 4NQO and arecoline *N*-oxide. Both were found to be mutagenic in *Salmonella* tester strains TA 98 and TA100; the carcinogenicity of arecoline *N*-oxide remains to be demonstrated (see Table 7). It is very encouraging that our preliminary results have shown that arecoline *N*-oxide is very effective in transforming several

Table 7. Attentation of 4NQO and Arecoline *N*-Oxide through Metabolic Inactivation by S9

	revertants (mean ± SD)[a]			
	TA 100		TA 98	
assay system	−S9	+S9	−S9	+S9
0.1 M phosphate buffer, pH 7.4	119 ± 8		8 ±	
S9 alone		130 ± 5		10 ± 2
4NQO (50 ng/plate)	631 ± 16	166 ± 7	58 ± 3	26 ± 2
4NQO (5 ng/plate)	252 ± 9	126 ± 4	29 ± 2	8 ± 2
4NQO (0.5 ng/plate)	133 ± 2	129 ± 3	23 ± 4	9 ± 1
arecoline *N*-oxide (1000 μg/plate)	716 ± 4	206 ± 12	90 ± 5	10 ± 2
arecoline *N*-oxide (100 μg/plate)	311 ± 5	121 ± 4	43 ± 3	14 ± 2
arecoline *N*-oxide (10 μg/plate)	155 ± 6	119 ± 4	31 ± 2	15 ± 1

[a] Data from the average of 3three determinations (mean ± SD), SD for standard deviation.

primary oral fibroblast and keratinocyte cell lines (Lee et al., unpublished observation).[33] The development of this kind of tumor has been considered in persons with a high risk of oral cancer such as smokers or alcohol consumers as well as patients diagnosed with oral dysplasia or carcinoma.[32] The mutagenicity of 4NQO had been reported.[26]

The relationship between arecoline *N*-oxide and clinical observation of the above experimental results is presented in Figure 2. It is very encouraging to find that the effective concentration of arecoline *N*-oxide might be generated in the saliva or blood of areca nut chewing people. It is proposed that arecoline *N*-oxide might play an important role in the development of oral carcinogensis in the areca nut chewing populations; meanwhile, a fraction of arecoline *N*-oxide may be eliminated by reducing compounds such as SH compounds (see Figure 2) and NADPH. In the future study, more experimental data on the carcinogenicity of arecoline *N*-oxide in mammalian systems should be provided to support this proposal. Further investigation on transformation of arecoline *N*-oxide in mammalian cells and animal models are in progress. It is encouraging to report that arecoline *N*-oxide was active in transforming several normal oral keratinocytes and inducing oral cancer nodules in hamster buccal pouch in 3 months.

Possible Carcinogens in Betel Quid (BQ). Several constituents in betel quid may be considered as possible candidates of carcinogens that may induce oral carcinogenesis in areca-chewing humans. These constituents were arecaline, arecaidine, guavanine, quavine, etc. Both arecaline and arecaidine have been shown to cause cell transformation in vitro[34] and chromosomal aberrations in mice,[2] but the carcinogenicity of these two compounds has never been demonstrated. BQ is a popular form of product for areca nut consumption. BQ has been made from areca nut, piper betle leaf, and alkali lime. It has been reported that piper betel leaf contained some safarole, a weak hepatic carcinogen in rodent species,[11] but areca nut did not contain safrole.[35] Recently, the areca nut alone was consumed by areca-chewing people.

AUTHOR INFORMATION

Corresponding Author

*Postal address: Institute of Biochemistry and Molecular Biology, College of Medicine, National Taiwan University, No. 1, Section 1, Jen-ai Road, Taipei, Taiwan. Phone: (886)-2-2356-2213. Fax: (886)-2-2391-8944. E-mail: jklin@ha.mc.ntu.edu.tw.

Funding Sources

This study was supported by the National Science Council NSC98-2313-B-002- 048 and NSC97-2321-B-002-016.

ACKNOWLEDGMENT

We thank Dr. Hsiou-Chen Huang for her assistance in NMR analysis. We thank Dr. M. H. Pan of the Department of Seafood Science, National Kaohsiung Marine University, for his kind assistance in revising the format of references and tables.

REFERENCES

(1) Gupta, P. C.; Warnakulasuriya, S. Global epidemiology of areca nut usage. *Addict. Biol.* **2002**, *7*, 77–83.

(2) Betel-quid and areca-nut chewing and some areca-nut derived nitrosamines. *IARC Monogr. Eval. Carcinog. Risks Hum.* **2004**, *85*, 1−334.

(3) Bhargava, K.; Smith, L. W.; Mani, N. J.; Silverman, S., Jr.; Malaowalla, A. M.; Bilimoria, K. F. A follow up study of oral cancer and precancerous lesions in 57,518 industrial workers of Gujarat, India. *Indian J. Cancer* **1975**, *12*, 124–129.

(4) Chang, K. M. Betel nut chewing and mouth cancer in Taiwan. First report: survey of disposition of mouth cancer in Taiwan. *Tsa Chih Gaoxiong Yi Xue Yuan Tong Xue Hui* **1964**, *63*, 437–448.

(5) Warnakulasuriya, S.; Trivedy, C.; Peters, T. J. Areca nut use: an independent risk factor for oral cancer. *BMJ* **2002**, *324*, 799–800.

(6) Chiu, C. J.; Chang, M. L.; Chiang, C. P.; Hahn, L. J.; Hsieh, L. L.; Chen, C. J. Interaction of collagen-related genes and susceptibility to betel quid-induced oral submucous fibrosis. *Cancer Epidemiol. Biomarkers Prev.* **2002**, *11*, 646–653.

(7) Hung, H. C.; Chuang, J.; Chien, Y. C.; Chern, H. D.; Chiang, C. P.; Kuo, Y. S.; Hildesheim, A.; Chen, C. J. Genetic polymorphisms of CYP2E1, GSTM1, and GSTT1; environmental factors and risk of oral cancer. *Cancer Epidemiol. Biomarkers Prev.* **1997**, *6*, 901–905.

(8) Ko, Y. C.; Huang, Y. L.; Lee, C. H.; Chen, M. J.; Lin, L. M.; Tsai, C. C. Betel quid chewing, cigarette smoking and alcohol consumption related to oral cancer in Taiwan. *J. Oral Pathol. Med.* **1995**, *24*, 450–453.

(9) Lu, C. T.; Yen, Y. Y.; Ho, C. S.; Ko, Y. C.; Tsai, C. C.; Hsieh, C. C.; Lan, S. J. A case-control study of oral cancer in Changhua County, Taiwan. *J. Oral Pathol. Med.* **1996**, *25*, 245–248.

(10) Miller, J. A. Recent studies on the metabolic activation of chemical carcinogens. *Cancer Res.* **1994**, *54*, 1879s–1881s.

(11) Miller, J. A. Carcinogenesis by chemicals: an overview − G. H. A. Clowes memorial lecture. *Cancer Res.* **1970**, *30*, 559–576.

(12) Ames, B. N.; Durston, W. E.; Yamasaki, E.; Lee, F. D. Carcinogens are mutagens: a simple test system combining liver homogenates for activation and bacteria for detection. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 2281–2285.

(13) Guengerich, F. P. Metabolism of chemical carcinogens. *Carcinogenesis* **2000**, *21*, 345–351.

(14) Maron, D. M.; Ames, B. N. Revised methods for the *Salmonella* mutagenicity test. *Mutat. Res.* **1983**, *113*, 173–215.

(15) Callander, R. D.; Mackay, J. M.; Clay, P.; Elcombe, C. R.; Elliott, B. M. Evaluation of phenobarbital/β-naphthoflavone as an alternative S9-induction regime to Aroclor 1254 in the rat for use in in vitro genotoxicity assays. *Mutagenesis* **1995**, *10*, 517–522.

(16) Nery, R. The metabolic interconversion of arecoline and arecoline 1-oxide in the rat. *Biochem. J.* **1971**, *122*, 503–508.

(17) Garner, R. C.; Miller, E. C.; Miller, J. A. Liver microsomal metabolism of aflatoxin B 1 to a reactive derivative toxic to *Salmonella typhimurium* TA 1530. *Cancer Res.* **1972**, *32*, 2058–2066.

(18) Giri, S.; Idle, J. R.; Chen, C.; Zabriskie, T. M.; Krausz, K. W.; Gonzalez, F. J. A metabolomic approach to the metabolism of the areca

nut alkaloids arecoline and arecaidine in the mouse. *Chem. Res. Toxicol.* **2006**, *19*, 818–827.

(19) Vogel, H. J.; Bonner, D. M. Acetylornithinase of *Escherichia coli*: partial purification and some properties. *J. Biol. Chem.* **1956**, *218*, 97–106.

(20) Gomori, G. Preparation of buffers for use in enzyme studies. *Methods Enzymol.* **1955**, *1*, 138–146.

(21) Jin, Y. T.; Tsai, S. T.; Wong, T. Y.; Chen, F. F.; Chen, R. M. Studies on promoting activity of Taiwan betel quid ingredients in hamster buccal pouch carcinogenesis. *Eur. J. Cancer B: Oral Oncol.* **1996**, *32B*, 343–346.

(22) Saraswathi, T. R.; Sheeba, T.; Nalinkumar, S.; Ranganathan, K. Effect of glutathione on arecanut treated normal human buccal fibroblast culture. *Indian J. Dent. Res.* **2006**, *17*, 104–110.

(23) Chiang, C. P.; Chang, M. C.; Lee, J. J.; Chang, J. Y.; Lee, P. H.; Hahn, L. J.; Jeng, J. H. Hamsters chewing betel quid or areca nut directly show a decrease in body weight and survival rates with concomitant epithelial hyperplasia of cheek pouch. *Oral Oncol.* **2004**, *40*, 720–727.

(24) Kataoka, K.; Kinouchi, T.; Ohnishi, Y. Species differences in metabolic activation and inactivation of 1-nitropyrene in the liver. *Cancer Res.* **1991**, *51*, 3919–3924.

(25) Giri, S.; Krausz, K. W.; Idle, J. R.; Gonzalez, F. J. The metabolomics of (±)-arecoline 1-oxide in the mouse and its formation by human flavin-containing monooxygenases. *Biochem. Pharmacol.* **2007**, *73*, 561–573.

(26) Lin, J. K.; Kuo, M. L.; Hsu, L. W. *Studies on the Genotoxicity of Dipyridyl and Biphenyl Derivatives Using Salmonella typhimurium*, 3rd ed.; 1988; pp 161−171.

(27) Kulanthaivel, P.; Barbuch, R. J.; Davidson, R. S.; Yi, P.; Rener, G. A.; Mattiuz, E. L.; Hadden, C. E.; Goodwin, L. A.; Ehlhardt, W. J. Selective reduction of *N*-oxides to amines: application to drug metabolism. *Drug Metab. Dispos.* **2004**, *32*, 966–972.

(28) Zhang, X.; Reichart, P. A. A review of betel quid chewing, oral cancer and precancer in Mainland China. *Oral Oncol.* **2007**, *43*, 424–430.

(29) Strickland, S. S.; Veena, G. V.; Houghton, P. J.; Stanford, S. C.; Kurpad, A. V. Areca nut, energy metabolism and hunger in Asian men. *Ann. Hum. Biol.* **2003**, *30*, 26–52.

(30) Tanaka, T.; Kohno, H.; Sakata, K.; Yamada, Y.; Hirose, Y.; Sugie, S.; Mori, H. Modifying effects of dietary capsaicin and rotenone on 4-nitroquinoline 1-oxide-induced rat tongue carcinogenesis. *Carcinogenesis* **2002**, *23*, 1361–1367.

(31) Nair, J.; Ohshima, H.; Friesen, M.; Croisy, A.; Bhide, S. V.; Bartsch, H. Tobacco-specific and betel nut-specific *N*-nitroso compounds: occurrence in saliva and urine of betel quid chewers and formation in vitro by nitrosation of betel quid. *Carcinogenesis* **1985**, *6*, 295–303.

(32) Silva, R. N.; Ribeiro, D. A.; Salvadori, D. M.; Marques, M. E. Placental glutathione *S*-transferase correlates with cellular proliferation during rat tongue carcinogenesis induced by 4-nitroquinoline 1-oxide. *Exp. Toxicol. Pathol.* **2007**, *59*, 61–68.

(33) Ohne, M.; Satoh, T.; Yamada, S.; Takai, H. Experimental tongue carcinoma of rats induced by oral administration of 4-nitroquinoline 1-oxide (4NQO) in drinking water. *Oral Surg. Oral Med. Oral Pathol.* **1985**, *59*, 600–607.

(34) Merchant, A.; Husain, S. S.; Hosain, M.; Fikree, F. F.; Pitiphat, W.; Siddiqui, A. R.; Hayder, S. J.; Haider, S. M.; Ikram, M.; Chuang, S. K.; Saeed, S. A. Paan without tobacco: an independent risk factor for oral cancer. *Int. J. Cancer* **2000**, *86*, 128–131.

(35) Jeng, J. H.; Kuo, M. L.; Hahn, L. J.; Kuo, M. Y. Genotoxic and non-genotoxic effects of betel quid ingredients on oral mucosal fibroblasts in vitro. *J. Dent. Res.* **1994**, *73*, 1043–1049.