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Metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) by human cytochrome P450 1A2 and its inhibition by phenethyl isothiocyanate

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4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is a potent tobacco-specific nitrosamine in animals and has been suggested to play a role in human tobacco-related cancers. Our previous study demonstrated that cytochrome P450 (P450) 1A2 catalyzes the formation of 4-hydroxy-1-(3-pyridyl)-1-butanone (keto alcohol) (an α-hydroxylation product) from NNK in human liver microsomes. Phenethyl isothiocyanate (PEITC) inhibits NNK tumorigenesis by blocking the activation of NNK. The purpose of the present study was to elucidate the mechanism of inhibition of P450 1A2-catalyzed NNK activation by PEITC. Human P450 1A2 was expressed in *Escherichia coli* and purified to homogeneity. In a reconstituted system, P450 1A2 catalyzed the formation of keto alcohol and 4-oxo-1-(3-pyridyl)-1-butanone (keto aldehyde) from NNK, with the keto alcohol being the major metabolite. The apparent K_m and V_{max} values for keto alcohol formation was 380 μM and 1.7 nmol/min/nmol P450, respectively. For the tobacco-specific nitrosamine *N*-nitrosonornicotine (NNN), P450 1A2 catalyzed the formation of the derived 4-hydroxy-4-(3-pyridyl)butyric acid (hydroxy acid), 4-oxo-4-(3-pyridyl)butyric acid (keto acid) and keto alcohol. In comparison to NNK, NNN had a lower rate of oxidation with P450 1A2. PEITC decreased the formation of the NNK-derived keto alcohol in a concentration-dependent manner, with an IC_{50} value of 0.14 μM. PEITC was a competitive inhibitor of P450 1A2, exhibiting a K_i value of 0.18 μM. Preincubation of PEITC with NADPH in the reconstituted system resulted in a further decrease (25%) in the catalytic activity of P450 1A2, suggesting that there is a slow metabolism-dependent inhibition of P450 1A2 by PEITC. The formation of keto aldehyde and keto alcohol was inhibited by PEITC in human liver microsomes with IC_{50} values of 9.5 and 4.6 μM respectively. Methoxyresorufin *O*-dealkylase activity, a marker for P450 1A2, was decreased by PEITC in a concentration-dependent manner, with an IC_{50} of 0.34 μM. The results suggest that PEITC itself is a potent inhibitor of P450 1A2 and that a metabolite(s) of PEITC can also inhibit P450 1A2. We conclude that PEITC may be an effective inhibitor of the carcinogenicity or toxicity of chemicals that are activated by P450 1A2.

Introduction

Tobacco and tobacco smoke contain several classes of carcinogens, such as polycyclic aromatic hydrocarbons and nitrosamines, and is one of the most important causes of cancer (1). 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK*), a potent tobacco-specific nitrosamine formed from the nitrosation of nicotine, induces lung, liver, nasal cavity and exocrine pancreatic tumors in rodents and has been suggested to play a role in human tobacco-related cancers (2,3). Like most carcinogens, NNK requires metabolic activation to form reactive intermediates which can interact with cellular nucleophiles. Metabolic activation of NNK involves α-hydroxylation of either the methylene or methyl carbon of NNK, resulting in the methylation or pyridyloxobutylation of DNA respectively (2). Cytochrome P450 (P450) enzymes have been shown to play a major role in the activation process (4–8). Although NNK can be activated by more than one P450 enzyme, P450 1A2 appears to have an important role in the activation of NNK. Our previous study demonstrated that human P450 1A2 expressed in Hep G2 cells had the highest activity in catalyzing the formation of 4-hydroxy-1-(3-pyridyl)-1-butanone (keto alcohol) (an α-hydroxylation product) and P450 1A2 was a major enzyme involved in the activation of NNK in human liver microsomes (6). Crespi *et al.* (9) showed that P450 1A2 expressed in a B-lymphoblastoid cell line enhanced NNK-induced mutagenicity and cytotoxicity. Furthermore, P450 1A2 has been shown to be the major enzyme in human liver microsomes that catalyses the bioactivation of genotoxic components of cigarette smoke condensate (10,11).

Epidemiological studies have shown that frequent consumption of fruits and vegetables is associated with lower risks of various cancers (12,13). Phenethyl isothiocyanate (PEITC), a naturally occurring compound present in cruciferous vegetables, is the myrosinase-catalyzed product of gluconasturtiin (a glucosinolate) (14). Gluconasturtiin is efficiently converted to PEITC in humans and the major route for excretion of PEITC is the urine (15,16). PEITC effectively inhibited NNK-induced DNA adduct formation and lung tumorigenesis in animals (17,18). However, PEITC must be given before the carcinogen in order to inhibit NNK tumorigenesis (19), suggesting that the inhibitory activity of PEITC is due to blocking the activation of NNK. PEITC inhibits the bioactivation of NNK by chemical inactivation and competitive inhibition of the enzyme(s) responsible for its oxidation (4,8) and is a selective inhibitor of P450 enzymes (20–22). In contrast to the inhibitory activity of PEITC against NNK-induced lung tumorigenesis, PEITC had no effect on benzo[*a*]pyrene-induced lung tumorigenesis and DNA adduct formation in mice (23). This differential biological effect of PEITC may

*Abbreviations: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; P450, cytochrome P450; keto alcohol, 4-hydroxy-1-(3-pyridyl)-1-butanone; PEITC, phenethyl isothiocyanate; NNN, *N*-nitrosonornicotine; HPLC, high performance liquid chromatography; DMSO, dimethyl sulfoxide; MROD, methoxyresorufin *O*-dealkylase; keto aldehyde, 4-oxo-1-(3-pyridyl)-1-butanone; hydroxy acid, 4-hydroxy-4-(3-pyridyl)butyric acid; keto acid, 4-oxo-4-(3-pyridyl)butyric acid; IC_{50}, inhibitor concentration resulting in 50% inhibition of the activity; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol.

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be due to different enzymes being involved in the activation of NNK and benzo[*a*]pyrene in mouse lung. Since P450 1A2 is one of the major enzymes that activates NNK in humans and PEITC is a potential chemopreventive agent, it is important to determine if PEITC is an effective inhibitor of human P450 1A2. In the present study, the role of human P450 1A2 in the activation of NNK and the mechanism of inhibition by PEITC were determined in a reconstituted system.

Materials and methods

Chemicals

Unlabeled NNK, *N*-nitrosonornicotine ([^{3}H]NNN) (3.4 Ci/mmol; purity >95%) and [5-^{3}H]NNK (2.4 Ci/mmol; purity >97%) were purchased from Chemsyn Science Laboratories (Lenexa, KS). The radiolabeled NNN and NNK were further purified by reverse-phase high pressure liquid chromatography (HPLC) before use. NNK metabolite standards were kindly supplied by Dr Stephen Hecht of the American Health Foundation (Valhalla, NY). PEITC (purity 99%) was from Aldrich Chemical Co. (Milwaukee, WI). L-α-Dilauroylphosphatidylcholine, DMSO, NADPH, NADP$^+$, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were purchased from Sigma Chemical Co. (St Louis, MO). Resorufin and methoxyresorufin were obtained from Pierce Chemical Co. (Rockford, IL). All other chemicals were of reagent grade.

P450 1A2 reconstituted system

Human P450 1A2 was expressed in *Escherichia coli* and purified to homogeneity as described (24). NADPH-P450 reductase was purified from liver microsomes of phenobarbital-treated rabbits (25) with slight modifications (26). For the reconstituted system, P450 1A2, reductase and dilauroylphosphatidylcholine (sonicated briefly prior to use) were mixed together and allowed to stand at room temperature for 5 min before addition to incubations.

Human liver microsomes

A human liver sample (HL 114) was obtained from the Tennessee Donor Services (Nashville, TN; coded 'HL' for human liver and a code number), and microsomes were prepared (27) and stored at −80°C Glycerol in the microsomal preparation was removed by dialysis against 0.25 M sucrose before use.

NNK metabolism analysis

Unless otherwise specified, incubations consisted of 100 mM Tris−HCl (pH 7.4), 1 mM EDTA, 20 mM $MgCl_2$, 30 mM KCl, an NADPH-generating system (5 mM glucose 6-phosphate, 1 mM NADP$^+$ and 1.5 units glucose 6-phosphate dehydrogenase), 10 μM NNK (containing 1.0 μCi [5-^{3}H]NNK), 0.05 nmol P450 1A2, 0.15 nmol reductase and 30 μM dilauroylphosphatidylcholine in a total volume of 0.4 ml. Reactions were carried out at 37°C for 30 min and terminated with 100 μl each of 25% $ZnSO_4$ and saturated $Ba(OH)_2$. Each sample was centrifuged and filtered, and 0.2 ml was coinjected with 5 μl of NNK metabolite standards onto a reverse-phase HPLC system equipped with a radioflow detector (Radiomatic Instruments and Chemical Co., Tampa, FL) (4). The HPLC conditions used were the same as previously described (7). When $NaHSO_3$ (5 mM) was added to the incubation mixture to trap 4-oxo-1-(3-pyridyl)-1-butanone (keto aldehyde) (28), the metabolism of NNK was inhibited by 50%. Therefore, $NaHSO_3$ was added only at the end of the incubation. To incorporate PEITC into the incubation mixture, PEITC was dissolved in DMSO; the final concentration of DMSO in the incubation was 0.125% (v/v). At this concentration, DMSO did not affect NNK metabolism.

MROD analysis

MROD activity was assayed as described previously for ethoxyresorufin *O*-dealkylase activity (20) except that MROD (5 μM) was used as the substrate. The enzyme activities were quantitated by comparison to a resorufin standard curve subjected to the same incubation procedure. All data were statistically analyzed by analysis of variance.

Results

Metabolism of NNK and NNN by human P450 1A2

In a reconstituted system, P450 1A2 catalyzed the formation of the keto aldehyde and keto alcohol, with keto alcohol being the major metabolite. At an NNK concentration of 10 μM, the rate of keto alcohol formation (45.5 pmol/min/nmol P450) was ~9-fold higher than that of the keto aldehyde (4.8 pmol/min/nmol P450), showing that P450 1A2 preferentially catalyzes the methyl hydroxylation of NNK. The rate of formation of keto alcohol was linear with time (data not shown). When cytochrome b_5 was added to the reconstituted system at a P450:cytochrome b_5 ratio of 1:4, the formation of keto aldehyde and keto alcohol decreased by 50 and 80% respectively (data not shown).

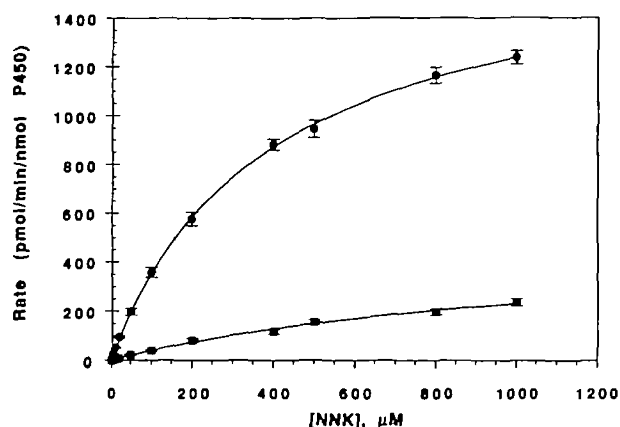

Fig. 1. Dependency on substrate concentration for the formation of keto alcohol and keto aldehyde by P450 1A2. Incubations contained 0.05 nmol P450 1A2, NADPH-generating system and 1–1000 μM NNK containing 1.0 μCi [5-^{3}H]NNK. Reactions were carried out for 30 min at 37°C, and formation of the keto aldehyde (■) and keto alcohol (●) was determined. Each point is the mean ± SD of three replicates.

The metabolism of NNN by P450 1A2 resulted in the formation of 4-hydroxy-4-(3-pyridyl)butyric acid (hydroxy acid), keto acid and keto alcohol. However, the activity was extremely low. At a concentration of 5 μM, the formation of keto alcohol from NNN was 2% of that from NNK (0.59 versus 25.8 pmol/min/nmol P450).

Substrate dependency for NNK activation

Michaelis−Menten kinetics were observed for P450 1A2 catalysed keto alcohol and keto aldehyde formation in the substrate concentration range of 1–1000 μM (Figure 1). The apparent K_m and V_{max} values (mean±SD) for keto alcohol formation were 380±30 μM and 1.7±0.05 nmol/min/nmol P450 respectively. For keto aldehyde formation, however, the apparent K_m and V_{max} values were 1180±60 μM and 0.51±0.04 nmol/min/nmol P450 respectively.

Inhibition of NNK metabolism by PEITC

Since the above results suggest that P450 1A2 is an important enzyme in catalyzing the formation of NNK-derived keto alcohol, the effect of PEITC on the formation of this metabolite was studied. The addition of PEITC (0–10 μM) to incubations decreased the rate of formation of the keto alcohol by P450 1A2 in a concentration-dependent manner (Figure 2). The rate of formation of the keto alcohol was decreased by 90% with 1.0 μM PEITC. The estimated IC_{50} was 0.14 μM.

PEITC was added to incubations containing P450 1A2 and different concentrations of NNK. The mechanism of inhibition of the keto alcohol formation by PEITC was competitive (Figure 3); in the presence of PEITC, the K_m significantly increased while the V_{max} was not appreciably affected (Table I). PEITC displayed an apparent K_i value of 0.18 μM.

In order to elucidate further the mechanisms of the inhibitory action of PEITC, a two-stage incubation was carried out in which P450 1A2 was preincubated with PEITC in the presence or absence of NADPH during the first stage. Aliquots were taken at different time points and diluted 20-fold in the second

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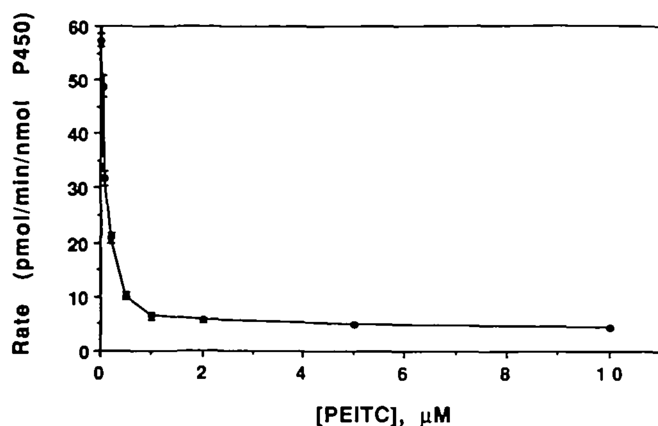

Fig. 2. Concentration-dependent inhibition of keto alcohol formation by PEITC. Incubations contained 0.05 nmol P450 1A2, NADPH-generating system, 400 μM NNK and 0–10 μM PEITC. Reactions were carried out for 30 min at 37°C. The points are means ± SD of four determinations.

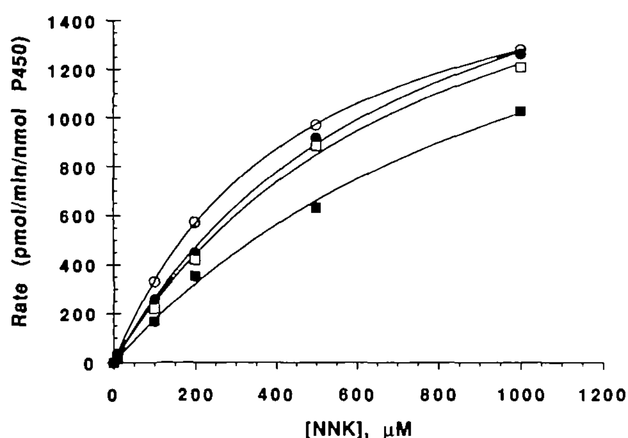

Fig. 3. Inhibition of keto alcohol formation by PEITC in a P450 1A2 reconstituted system. Incubations contained 0.05 nmol P450 1A2, 10–1000 μM NNK, 1.0 mM NADPH and 0 (○), 0.05 (●), 0.10 (□) or 0.20 (■) μM PEITC. Incubations were carried out for 15 min at 37°C. The points are the means of four determinations. The difference between the determinations was <10%.

Table I. Kinetic parameters of keto alcohol formation and its inhibition by PEITC[a]

Condition	K_m (μM)	V_{max} (nmol/min/nmol P450)
DMSO	400±10[b,1]	1.96±0.07[1]
0.05 μM PEITC	760±10[2]	2.09±0.20[1]
0.10 μM PEITC	820±20[2]	2.06±0.21[1]
0.20 μM PEITC	1240±70[3]	2.05±0.25[1]

[a]Incubations contained 0.05 nmol P450 1A2, 10–1000 μM NNK, 1 mM NADPH and 0–0.20 μM PEITC and were carried out at 37°C for 15 min.
[b]Values are the mean ± SD of four determinations. Values in the same column with different numerical superscripts are significantly ($P < 0.05$) different from each other.

stage incubation. Over a 30 min time period in which PEITC was preincubated in the absence of NADPH, there was no decrease in the rate of formation of the keto alcohol, suggesting that PEITC is not inactivating P450 1A2 by direct reaction with the P450 (Figure 4). However, when PEITC was preincubated in the presence of NADPH there was a slow metabolism-dependent decrease in the formation of keto alcohol over time. With a 30 min preincubation, the activity was

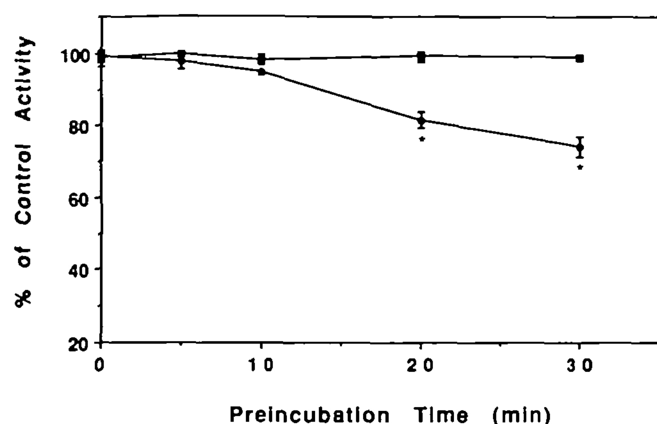

Fig. 4. Time-dependent inhibition of keto alcohol formation by PEITC. Reaction mixtures containing 0.05 nmol P450 1A2 and 0.60 μM PEITC in the presence (●) and absence (■) of 1.0 mM NADPH were preincubated for 0, 5, 10, 20 and 30 min at 37°C. The respective controls were P450 1A2 + DMSO + NADPH and P450 1A2 + DMSO and they were subjected to the same preincubation time periods. An aliquot of the reaction mixtures was then added to test tubes (20-fold dilution) containing 400 μM NNK, buffer and 1.0 mM NADPH and reactions were carried out for 10 min at 37°C. Points are the means ± SD of four determinations. Values with an asterisk (*) are significantly ($P < 0.05$) different from other time points.

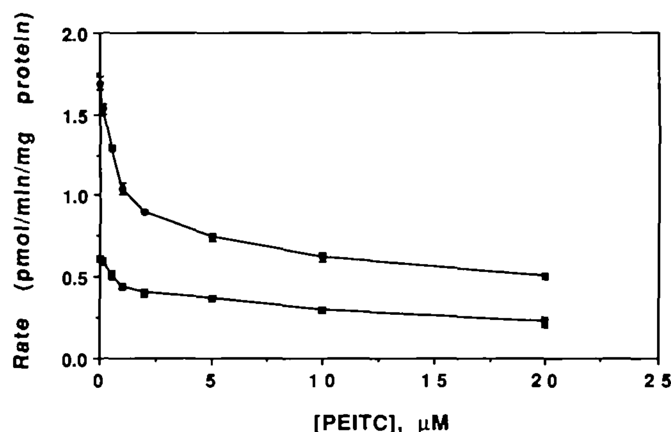

Fig. 5. Inhibition by PEITC of keto aldehyde and keto alcohol formation in human liver microsomes. Incubations contained 10 μM NNK, NADPH-generating system, 5 mM $NaHSO_3$, 0.10 mg microsomal protein and 0–20 μM PEITC in a total volume of 0.4 ml. PEITC was dissolved in methanol and this solution was used at 0.5% of the total incubation volume. Reactions were carried out at 37°C for 30 min and the formation of keto aldehyde (■) and keto alcohol (●) were determined. Each point is the mean ± SD of four determinations.

significantly decreased by 25% (Figure 4), suggesting that a product(s) of PEITC is inactivating P450 1A2. These results were confirmed when P450 1A2 was preincubated with 0.10 μM PEITC; inactivation of P450 1A2 was observed in the presence but not in the absence of NADPH (data not shown).

The inhibitory action of PEITC on NNK metabolism was further investigated in human liver microsomes (sample HL 114). This human liver sample is known to contain P450 1A2 (6). NNK metabolism in the human liver microsomes resulted in formation of the keto aldehyde, keto alcohol and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL). As the PEITC concentration increased from 0 to 20 μM, there was a progressive decrease in the rate of formation of the keto aldehyde and keto alcohol (Figure 5). The estimated IC_{50} values were 9.5 μM for keto aldehyde and 4.6 μM for keto alcohol formation. PEITC had no effect on NNAL formation

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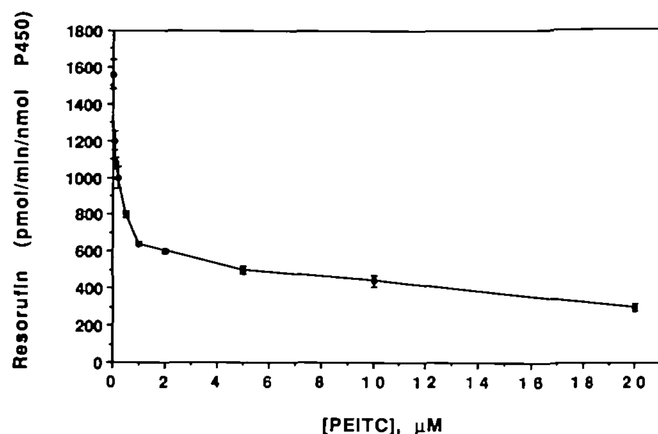

Fig. 6. Concentration-dependent inhibition of resorufin production from methoxyresorufin by PEITC. Incubations contained 0.025 nmol P450 1A2, 0.075 nmol NADPH-P450 reductase, 30 μM dilauroylphosphatidylcholine, 5 μM methoxyresorufin, NADPH-generating system and 0–20 μM PEITC Reactions were carried out at 37°C for 10 min. Values are means ± SD of four determinations.

(data not shown). The results suggest that PEITC inhibits P450 1A2 as well as other P450s present in the liver microsomes.

Metabolism of MROD and the effect of PEITC

MROD is a marker for P450 1A2 activity. In a reconstituted system, P450 1A2 catalyzed the formation of resorufin from 5.0 μM MROD at a rate of 1.6 nmol/min/nmol P450. PEITC decreased MROD activity in a concentration-dependent manner, exhibiting an IC_{50} of 0.34 μM (Figure 6). These results provide further evidence for the potent inhibitory activity of PEITC for P450 1A2.

Discussion

P450 1A2 is one of the major P450 enzymes present in human liver where it is inducible by cigarette smoking and the consumption of charbroiled foods (29). The present study demonstrates that P450 1A2 catalyzes the formation of both the keto aldehyde and keto alcohol from NNK. However, the very low activity and high K_m (1180 μM) in catalyzing the formation of keto aldehyde indicate that P450 1A2 is not an important enzyme for the α-methylene hydroxylation of NNK, a pathway that can lead to DNA methylation. On the other hand, P450 1A2 had high catalytic activity for the formation of the keto alcohol. The α-methyl hydroxylation of NNK can lead to the pyridyloxobutylation of DNA, which has been suggested to be important in NNK carcinogenesis and mutagenicity (3,30). The apparent K_m of 380 μM observed for keto alcohol formation is similar to that obtained previously with P450 1A2 expressed in Hep G2 cells (6). Human liver microsomes also displayed an apparent K_m value of ~400 μM for keto alcohol formation (6), similar to that observed for P450 1A2. With rodent microsomes, however, much lower K_m values of 2–29 μM have been observed (4,5,8).

NNK and NNN are tobacco-specific nitrosamines that induce tumors in different target organs. NNK primarily induces lung tumors, whereas NNN induces esophageal and nasal cavity tumors in rodents (2). In the activation of NNK and NNN, the formation of keto alcohol (and the pyridyloxobutylating species) is the pathway that the two nitrosamines have in common (2). Although P450 1A2 catalyzed the formation of the keto alcohol from NNK and NNN, the rate of formation was 44-fold higher for NNK. Furthermore, Yamazaki *et al.* (31) showed that 7,8-benzoflavone, a P450 1A inhibitor, decreased the activation of NNK but not that of NNN in human liver microsomes. These results suggest that enzymes other than P450 1A2 are important for the activation of NNN in humans. P450 2E1, for example, has been suggested to play an important role in the activation of NNN in human liver (31). The involvement of different enzymes in the activation of NNK and NNN may explain the susceptibility of the lung and esophagus to NNK and NNN tumorigenesis, respectively.

The present study clearly demonstrates the inhibitory activity of PEITC against human P450 1A2. PEITC is an extremely potent competitive inhibitor that appears to have high affinity for binding to the active site of P450 1A2. Furthermore, it appears that PEITC can be metabolized by P450 1A2 to a reactive intermediate, which then inactivates P450 1A2 (suicide inhibition). This metabolism-dependent inhibition of P450 1A2 appears to be a slow process. The identity of this reactive intermediate is presently unknown. Although PEITC did not chemically inactivate P450 1A2 in the present study, PEITC is known to be reactive towards amino, histidyl and cysteinyl groups of proteins (32). Our laboratory has conducted in-depth studies elucidating the mechanistic basis for the inhibitory activity of PEITC on NNK carcinogenesis in animals (4,8,20,21). Our *in vitro* studies with rodent lung and liver microsomes have shown PEITC to be a competitive inhibitor and to chemically inactivate the P450s responsible for NNK activation. However, the enzymes that activate NNK in rodents may not necessarily be the same enzymes as those in humans. Therefore, it is possible that PEITC may not exhibit the same inhibitory activity in humans as has been observed with animals, especially since PEITC is not a general P450 inhibitor (20–22).

The activation of NNK in a human liver microsomal sample was inhibited by PEITC (Figure 5). In this particular microsomal sample, P450s 1A2 and 2E1 are mainly responsible for catalyzing the formation of the keto alcohol (6). Because PEITC is also a competitive and suicide inhibitor of P450 2E1 (22), it is expected to inhibit the formation of the keto alcohol by inhibiting the activities of both P450s 1A2 and 2E1. The rate of formation of the keto aldehyde was also significantly decreased by PEITC beyond the contribution of P450s 1A2 and 2E1 (6). It has been shown that P450 2A6 can activate NNK (6,9,31) and recent studies have suggested that P450 2A6 catalyzes the formation of the keto aldehyde (Patten *et al.*, unpublished results). Whether PEITC is an inhibitor of P450 2A6 remains to be established. Besides being an inhibitor of hepatic P450s, PEITC is also an inhibitor of NNK oxidation in human lung microsomes, exhibiting an IC_{50} of 5 μM (data not shown). Although P450 1A2 is not present in human lung and enzymes other than P450s are also important in the activation of NNK in human lung (6), PEITC may be effective against tobacco-related lung cancer.

In conclusion, the present study demonstrates that PEITC is an effective inhibitor of P450 1A2-catalyzed activation of NNK. Since P450 1A2 also catalyzes the activation of other carcinogens, such as arylacetamides and food-derived heterocyclic amines (11,33), PEITC may protect against the carcinogenicity of these compounds by blocking their activation.

Acknowledgements

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