Pre-2005

Leclerc (1954), Daems (1969), Cecchini (1976), Mességué (1979), Aloysius (1983), Van Hellemont (1985), Holmes (1989), Phillips (1995), Newall et al. (1996), Wichtl (1997), Delfosse (1998), Hoendervanger et al. (1998), Shealy (1998), Vogel (1999), Peraira et al. (2001), Vermeulen (2002), Durafourd (2002), Thomson et al. (2002), Ames et al. (2002), Van de Velde (2004), Brown (2004-A to D), Keck et al. (2004), Rose et al. (2005), Gill et al. (2007) …

Glucosinolates, sulfur-containing volatile oils, are abundantly present in the vacuole of cruciferae (in especially high concentrations in watercress). Examples of studied glucosinolates are gluconasturtiine and sinigrine. – Van Damme et al. (2004), Verkerk et al. (2004), Newall et al. (1997)

After damage to the plant cell (i.e. chewing, cutting, …) glucosinolates are directly converted to isothiocyanates by hydrolysis by myrosinases. This way, gluconasturtiine in watercress is converted to phenylethylisothiocyanate (PEITC). – Holmes (1989), Newall et al. (1996), Wichtl (1997), Verkerk et al. (2004), Brown (2004-D)

Besides PEITC, other allylisothiocyanates are formed (phenyl- or methyltiopropylderivates; MSHITC, MSOITC). However, this is in smaller concentrations and studies are scarse. More than 75% of the total concentration of isothiocyanates formed is PEITC. – Newall et al. (1996), Verhelst et al. (2004)

Anticarcinogenic bioactivities of glucosinolate hydrolysis products: the mechanisms include altered estrogen metabolism, protection against reactive oxygen species, alterted detoxification by induction of phase II enzymes, decreased carcinogen activation by inhibition of phase I enzymes and slowed tumor growth and induction of apoptosis. – Keck et al. (2004)

Isothiocyanates induce apoptosis of pre-cancerous cells and tumor cells by activation of the caspase-8 pathway, potentiated by JNK1. The chemopreventive activity of isothyocianates is influenced by the isothiocyanate bioavailibility. – Thornally (2002)

Watercress is the richest natural source of PEITC. – Brown (2004-G)

Broccoli and watercress suppress matrix mettalloproteinase and invasiveness of human breast cancer cells. – Rose et al. (2005)

Ingestion of an isothiocyanate metabolite from cruciferous vegetables (i.e. PEITC in watercress) inhibits growth of human prostate cancer cell xenographs by apoptosis and cell cycle arrest. – Chiao et al. (2004)

N-acetylcysteine conjugate of phenylethylisothiocyanate enhances apoptosis in growth-stimulated human lung cells. – Yang et al. (2005)

Pre-2017

Avato et al. (2015), De Cock (2015), Fofaria et al. (2015), Abdull Razis et al. (2014), Yeh et al. (2014), Powolny et al. (2011), Sakao et al. (2015), Li Q et al. (2016), Su et al. (2015), Wang et al. (2014), Yasukawa et al. (2016), Casanova et al. (2013), Wagner et al. (2013) …

Several epidemiological studies have shown that in humans, high consumption of brassica vegetables (watercress is part of the brassica family) is inversely linked to cancer risk, with a particular chemoprotective effect against lung, stomach, colon and rectum carcinomas. – Avato et al. (2015)

Isothiocyanates have chemopreventive and chemotherapeutic activity against various tumor types in vitro as well as in preclinical animal models. Some isothiocyanates (BITC, PEITC) advance to clinical trials. – Fofaria et al. (2015)

PEITC modulates carcinogen-metabolising enzyme systems at doses reflecting human intake. – Abdull Razis et al. (2014)

PEITC inhibits cell growth and induces apoptosis in oral cancer cells such as oral squamous cell carcinoma cells with various p53 statuses. – Yeh et al. (2014)

In transgenic mouse models, dietary PEITC suppressed prostate adenocarcinoma progression by induction of autophagic cell death. – Powolny et al. (2011)

Cancer chemopreventive PEITC suppresses the CXCR4 chemokine receptor expression in prostate cancer cells in vitro as well as in vivo. CXCR4 downregulation may be an important pharmacodynamic biomarker of cancer chemopreventative isothiocyanates in prostate adenocarcinoma. – Sakao et al. (2015)

PEITC can enhance cisplatin-induced apoptosis via glutathionylation-dependent degradation of Mc1-1. Dietary intake of watercress may help reverse cisplatin resistance in biliary tract cancer patients. – Li Q et al. (2016)

PEITC possesses the potential ability to inhibit proliferation, induce apoptosis and arrest cell cycling against LN229 human glioma cells related to the fact that PEITC can cause oxidative stress to tumor cells. – Su et al. (2015)

PEITC inhibits the growth of human chronic myeloid leukemia (CML) K562 cells, via caspases and the generation of reactive oxygen species (ROS). PEITC induces apoptosis in these cells. – Wang et al. (2014)

PEITC enters clinical trials as an inhibitor of metabolic activation of a tobacco-specific lung carcinogen in cigarette smokers. Overall, the NNK (toxin found in tobacco of cigarettes) metabolic activation was reduced by 7.7%. – Yuan et al. (2016)

Watercress juice did not induce genetic damage in male and female mice, exhibiting a protective activity against cyclophosphamide. The comparative analysis of bladder histological changes obtained in the watercress plus cyclophosphamide group against those treated with cyclophosphamide alone suggests a probably protective effect. Further studies are needed in order to establish the protective role of watercress juice against DNA damage. – Casanova et al. (2013)

Specific studies, compromising clinical trials in human subjects

1. Clinical Trial of 2-Phenethyl Isothiocyanate as an Inhibitor of Metabolic Activation of a Tobacco-Specific Lung Carcinogen in Cigarette Smokers

   Jian-Min Yuan,[1] Irina Stepanov,[3] Sharon E. Murphy,[3,4] Renwei Wang,[1] Sharon Allen,[3] Joni Jensen,[3] Lori Strayer,[3] Jennifer Adams-Haduch,[1] Pramod Upadhyaya,[3] Chap Le,[3] Mindy S. Kurzer,[5] Heather H. Nelson,[3,6] Mimi C. Yu,[7] Dorothy Hatsukami,[3] and Stephen S. Hecht - *Cancer Prev Res (Phila). 2016 May; 9(5): 396–405.*

   7.7% decrease in activation of carcinogen toxins after only a 1 week use of 40mg PEITC/day.

2. Phenylethyl isothiocyanate reverses cisplatin resistance in biliary tract cancer cells via glutathionylation-dependent degradation of Mcl-1

   Qiwei Li,[1] Ming Zhan,[1] Wei Chen,[1] Benpeng Zhao,[2] Kai Yang,[2] Jie Yang,[2] Jing Yi,[2] Qihong Huang,[3] Man Mohan,[2] Zhaoyuan Hou,[2] and Jian Wang – Oncotarget. 2016 Mar 1; 7(9): 10271–10282.

   This study highlights the potential efficacy of a combination cisplatin-watercress (PEITC).

Phenylethyl isothiocyanate reverses cisplatin resistance in biliary tract cancer cells via glutathionylation-dependent degradation of Mcl-1

Qiwei Li[1], Ming Zhan[1], Wei Chen[1], Benpeng Zhao[2], Kai Yang[2], Jie Yang[2], Jing Yi[2], Qihong Huang[3], Man Mohan[2], Zhaoyuan Hou[2], Jian Wang[1]

[1]Department of Biliary-Pancreatic Surgery, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China

[2]Department of Biochemistry and Molecular Cell Biology, Shanghai Key Laboratory of Tumor Microenvironment and Inflammation, Institutes of Medical Sciences, Shanghai Jiao Tong University, School of Medicine, Shanghai, China

[3]The Wistar Institute, University of Pennsylvania and Veterans Affairs Medical Center, Philadelphia, Pennsylvania, USA

Correspondence to: Jian Wang, **e-mail:** dr_wangjian@126.com

Keywords: *biliary tract cancer, PEITC, cisplatin, Mcl-1, glutathionylation*

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ABSTRACT

Biliary tract cancer (BTC) is a highly malignant cancer. BTC exhibits a low response rate to cisplatin (CDDP) treatment, and therefore, an understanding of the mechanism of CDDP resistance is urgently needed. Here, we show that BTC cells develop CDDP resistance due, in part, to upregulation of myeloid cell leukemia 1 (Mcl-1). Phenylethyl isothiocyanate (PEITC), a natural compound found in watercress, could enhance the efficacy of CDDP by degrading Mcl-1. PEITC-CDDP co-treatment also increased the rate of apoptosis of cancer stem-like side population (SP) cells and inhibited xenograft tumor growth without obvious toxic effects. *In vitro*, PEITC decreased reduced glutathione (GSH), which resulted in decreased GSH/oxidized glutathione (GSSG) ratio and increased glutathionylation of Mcl-1, leading to rapid proteasomal degradation of Mcl-1. Furthermore, we identified Cys16 and Cys286 as Mcl-1 glutathionylation sites, and mutating them resulted in PEITC-mediated degradation resistant Mcl-1 protein. In conclusion, we demonstrate for the first time that CDDP resistance is partially associated with Mcl-1 in BTC cells and we identify a novel mechanism that PEITC can enhance CDDP-induced apoptosis via glutathionylation-dependent degradation of Mcl-1. Hence, our results provide support that dietary intake of watercress may help reverse CDDP resistance in BTC patients.

INTRODUCTION

Biliary tract cancer (BTC) refers to a group of cancers of the biliary tract, including gallbladder cancer, cholangiocarcinoma of intrahepatic and extrahepatic bile ducts, and cancers of the ampulla and papilla of Vater [1]. BTC is a common form of cancer in East Asia and Latin America [2, 3] and surgical resection is the only curative treatment. However, most patients are diagnosed with advanced-stage disease, making them ineligible for complete surgical resection. The prognosis for patients with advanced BTC is very poor, and most survive for less than a year after diagnosis. Cisplatin (CDDP) based chemotherapy is widely used to treat patients with advanced BTC [4]. However, BTC cells are highly chemoresistant. The mechanism of CDDP resistance in BTC cells is poorly understood. Therefore, investigation into the mechanism of CDDP resistance and strategies to alleviate the CDDP resistance are urgently needed.

Various naturally occurring compounds are being tested for their anti-tumor activity. Such compounds when used in combination with known chemotherapeutic agents may help in overcoming chemoresistance, and may provide new strategies and ideas for treatment in clinic. Phenylethyl isothiocyanate (PEITC) is present in high concentrations as its precursor gluconasturtiin in cruciferous vegetables, such as watercress. Upon chewing or chopping, PEITC is released as a product of hydrolysis mediated by myrosinase [5]. Accumulating evidence indicates that PEITC can inhibit cell growth and

induce apoptosis in a variety of cancer cells, suggesting its potential value as an anticancer agent or an adjunctive therapy to current cancer treatment strategies [6–8]. Additionally, it was shown that eating watercress can significantly increase the blood level of PEITC in humans [9]. These studies and their results gave us important clue to study the effect of combined treatment with PEITC and CDDP on BTC cells.

Though recent data has shown that PEITC can sensitize some tumor cells to CDDP [5, 10–12], the synergistic effect of PEITC and CDDP in BTC cells has not been investigated. Here, we demonstrate for the first time that CDDP resistance is partially associated with Mcl-1 in BTC cells, and PEITC can enhance CDDP-induced apoptosis via glutathionylation-dependent degradation of myeloid cell leukemia 1 (Mcl-1). Interestingly and promisingly, PEITC-CDDP co-treatment can increase the rate of apoptosis of cancer stem-like side population (SP) cells and significantly reduce the growth of xenograft tumor without any major toxic effects. Our results suggests that dietary intake of watercress, which is a rich source of PEITC, may help reverse CDDP resistance in BTC patients.

RESULTS

PEITC enhances CDDP-induced inhibition of cell viability in BTC cells by increasing apoptosis

To examine the effect of PEITC-CDDP co-treatment on cell viability, human gallbladder cancer GBC-SD cells were treated with PEITC, CDDP, or a PEITC-CDDP combination, and cell metabolic activity was measured using the 3-(4, 5-dimethylthia-zol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay. Notably, PEITC-CDDP co-treatment led to a significant reduction in cell viability (Figure 1A). To quantify synergism, the median-drug effect analysis method was used and the Combination Index (CI) values were calculated. Synergism is indicated by a CI of less than 1, additivity by a CI equal to 1, and antagonism by a CI greater than 1. Normalised isobolograms (Figure 1B) display data points below the additivity line, indicating synergy in growth inhibition of GBC-SD cells. Drug Reduction Index (DRI) was then calculated (Table 1). These results suggest that PEITC-CDDP co-treatment possesses a synergistic effect on GBC-SD cell proliferation.

To determine whether PEITC in combination with CDDP decreases cell viability via an increase in apoptosis, annexin V-fluorescein isothiocyarate (Annexin V-FITC)/propidium iodide (PI) double labeling flow cytometry was used to determine the percentage of cells entering apoptosis. Flow cytometry analysis showed that CDDP caused about 10% of the cells to enter apoptosis, but co-treatment with PEITC dramatically enhanced CDDP-induced apoptosis to 40% (Figure 1C–1D). A similar pro-apoptotic effect of the combined treatment with PEITC and CDDP was also observed in human cholangiocarcinoma RBE cells (Figure 1E–1H). Together, these data demonstrated that PEITC can enhance CDDP-induced apoptosis in BTC cells.

PEITC enhances the sensitivity of SP cells and xenograft tumors to CDDP

Recent studies have shown that SP cells isolated from various cancer cell lines and primary tumors possess cancer stem-like properties [13–16]. SP cells can effectively avoid the effects of chemotherapeutic drugs, and are considered to be the root cause of tumor recurrence and metastasis. Therefore, we tested the effect of PEITC-CDDP co-treatment on SP cells from GBC-SD cells. The proportions of SP cells was 5.2% (Figure 2A). As shown in Figure 2B, flow cytometry analysis of SP cells treated with PEITC, CDDP, or a PEITC-CDDP combination showed that CDDP alone caused little cell apoptosis, but when combined with PEITC, markedly enhanced apoptosis at 24 hrs. These results demonstrate that PEITC significantly enhances the sensitivity of SP cells to CDDP.

To further examine the synergistic effect of PEITC and CDDP *in vivo*, GBC-SD cells were transplanted into nude mice. When the tumor size reached approximately 50 mm^3, mice were randomly sorted into four equal groups. The tumor-bearing mice were intra-peritoneally injected with physiological saline as a control, PEITC, CDDP or PEITC-CDDP combination for 10 days. Treatment of mice with CDDP alone moderately inhibited tumor growth, but PEITC-CDDP combination treatment resulted in a striking reduction in the average tumor weight by about 50% (Figure 2C). The potent *in vivo* anticancer effect in the PEITC-CDDP combined group was further evident in the tumor growth curve data (Figure 2D). Systemic toxic effects of the treatments in these mice were evaluated by measuring the loss in body weight. No notable differences were observed between the treated groups (Figure 2E). Collectively, these results demonstrate that PEITC-CDDP co-treatment can effectively inhibit tumor growth without obvious toxic effects *in vivo*.

Mcl-1 is partially responsible for CDDP resistance in GBC-SD and SP cells

Anti-apoptotic proteins such as B-cell lymphoma 2 (Bcl-2), B-cell lymphoma-extra large (Bcl-xl) and Mcl-1 are known to play key roles in cancer cell apoptosis. Therefore, we investigated the relationship between these anti-apoptotic proteins and CDDP resistance in GBC-SD cells. Interestingly, CDDP treatment increased Mcl-1 protein level in a time- and dose-dependent manner, but did not increase Bcl-2 or Bcl-xl protein amounts (Figure 3A). To determine if Mcl-1 is playing a role in the

cytotoxic sensitivity of GBC-SD cells to CDDP, cells were transfected with two siRNA oligonucleotides targeting Mcl-1 (Figure 3B), followed by CDDP treatment for 24 hrs. Knock down of Mcl-1 increased CDDP-induced apoptosis (Figure 3C). We also observed that Mcl-1 protein level was higher in SP cells than that in main population (MP) cells (Figure 3D). These data suggest that Mcl-1 is partially responsible for CDDP resistance in GBC-SD and SP cells.

PEITC enhances the cytotoxicity of CDDP through proteasomal degradation of Mcl-1 *in vitro* and *in vivo*

To better understand the mechanism of how PEITC overcomes CDDP resistance, we analyzed Mcl-1 protein level in lysates from GBC-SD cells treated with PEITC and/or CDDP. PEITC treatment decreased Mcl-1 expression in a time- and dose-dependent manner (Figure 4A–4B). Notably, combined treatment with PEITC and CDDP significantly decreased Mcl-1 protein level compared to CDDP alone (Figure 4C). In SP cells, the PEITC-CDDP treatment also lead to a significant decrease in Mcl-1 protein level compared to treatment only with CDDP (Figure 4D). To investigate the effect of PEITC-CDDP treatment on Mcl-1 protein level *in vivo*, tumor tissues harvested from two mice of each group were examined by immunoblotting. PEITC and/or CDDP-mediated changes in Mcl-1 protein level in the tumor tissue were generally in agreement with the molecular alteration observed in cultured cells (Figure 4E). Furthermore, exogenous overexpression of Mcl-1 impeded PEITC-CDDP-induced apoptosis (Figure 4F–4G). These data suggest that PEITC enhances the cytotoxicity of CDDP through a reduction in Mcl-1 *in vitro* and *in vivo*.

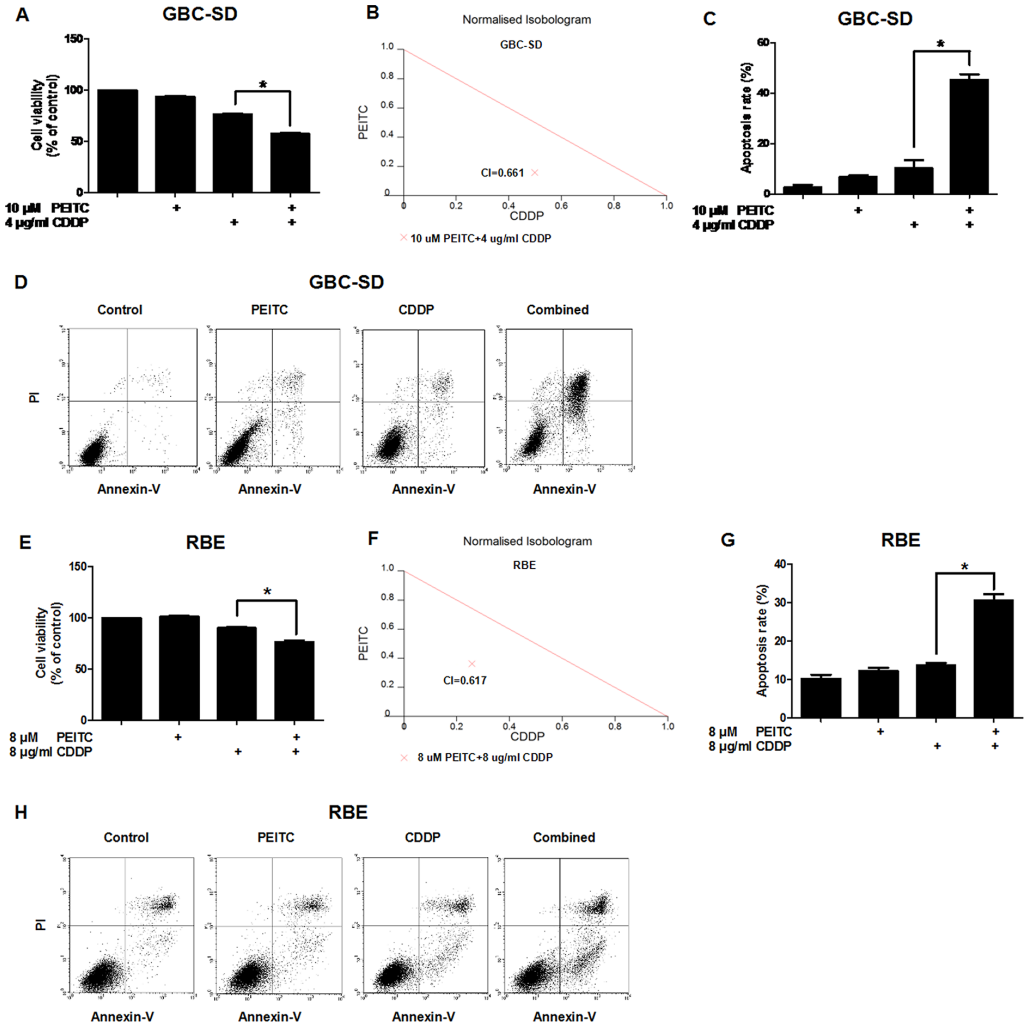

Figure 1: PEITC enhances CDDP-induced apoptosis in GBC-SD and RBE cells. (**A**) Cells were treated with PEITC, CDDP or PEITC-CDDP combination for 24 hrs and prepared for MTT assays. (**B**) CI of PEITC-CDDP treatment in GBC-SD cells. (**C**) Flow cytometry analysis of rate of apoptosis in GBC-SD cells (Annexin V/PI flow cytometry, bar charts). (**D**) Apoptosis in GBC-SD cells (density plots). (**E**) Cell viability assay of RBE cells (MTT). (**F**) CI in RBE cells. (**G**) Flow cytometry analysis of rate of apoptosis in RBE cells (Annexin V/PI flow cytometry, bar charts). (**H**) Apoptosis in RBE cells (density plots). Data shown is average of three independent experiments. *$P < 0.05$.

Table 1: CI and DRI values of PEITC and CDDP in BTC cells

Cell line	PEITC (uM)	CDDP (ug/ml)	Fa[a]	CI[b]	DRI[c] PEITC	DRI CDDP
GBC-SD	10	4	0.4282	0.661	6.157	2.006
RBE	8	8	0.2329	0.617	2.764	3.924

a. Fa: fractional inhibition.

b. CI < 1, = 1, and > 1 indicates synergism, additive effect, and antagonism, respectively.

c. DRI: fold of dose reduction for each drug in combination, for a given degree of inhibition, when compared with the dose of each drug alone for the same degree of inhibition. A DRI greater than 1 indicates an enhanced cytotoxicity for the combination.

To determine if the decrease in Mcl-1 protein level caused by PEITC was due to transcriptional inhibition or post-transcriptional regulation, we examined *Mcl-1* mRNA level by quantitative real time PCR in GBC-SD cells treated with PEITC. Surprisingly, PEITC increased *Mcl-1* mRNA level (Figure 4H). This suggested that PEITC-mediated decrease of Mcl-1 expression is regulated post-transcriptionally. Western blot analysis showed that Mcl-1 degradation was facilitated after 6 hours of PEITC treatment (Figure 4I). Next, to ask if PEITC mediated degradation of Mcl-1 involves proteasomal degradation, we treated GBC-SD cells with the proteasome inhibitor

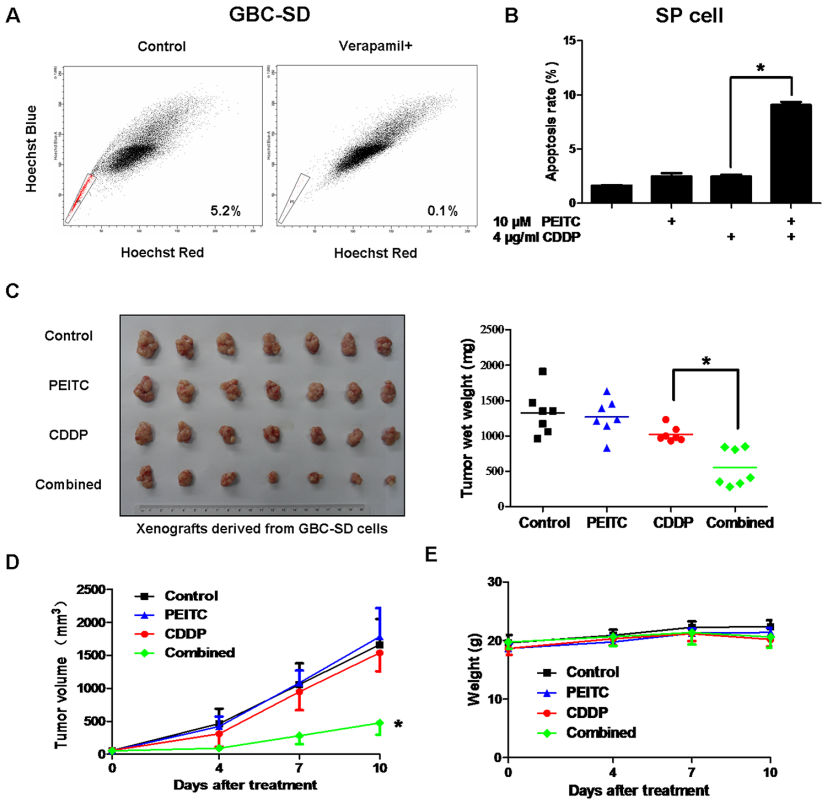

Figure 2: PEITC-CDDP co-treatment sensitizes SP cells and inhibits xenograft tumor growth without obvious toxic effects. (**A**) FACS analysis on single cell suspension of GBC-SD cells stained with Hoechst 33342 dye showing SP cells. SP cells are enclosed within the area demarcated in black. Verapamil inhibited the efflux of the dye and caused the disappearance of SP cells. A representative plot of the frequency of SP cells is provided. (**B**) SP cells from GBC-SD cells were treated with PEITC, CDDP or PEITC-CDDP combination for 24 hrs and apoptosis detected by Annexin V/PI assay. Data shown is average of three independent experiments. **P* < 0.05. (**C**) GBC-SD cells were transplanted into nude mice. When tumor size reached approximately 50 mm^3, mice were randomly sorted into four equal groups. The tumor-bearing mice were intra-peritoneally injected with physiological saline as a control, PEITC, CDDP or PEITC-CDDP combination for 10 days. Xenografts were excised and weighed. Each dot represents weight of one tumor, and the mean tumor weights of each group is indicated by solid lines (right panel; $n = 7$). **P* < 0.05. (**D**) Volume of the tumors was measured twice a week, and a tumor growth curve created for each group ($n = 7$). **P* < 0.05,. (**E**) Mice were weighed twice a week, and a weight curve created for each group ($n = 7$).

MG132 and found that the treatment recovered Mcl-1 protein amount to normal level (Figure 4J). Taken together, these data indicate that PEITC decreases Mcl-1 protein level via proteasomal degradation.

PEITC induces proteasomal degradation of Mcl-1 through depletion of reduced glutathione (GSH) and decrease of GSH/oxidized glutathione (GSSG) ratio

Previous studies have shown that PEITC can alter the redox state of cancer cells through GSH reduction [6, 7, 17]. Since Mcl-1 is a redox sensitive protein [7], we analyzed the relationship between GSH reduction and Mcl-1 degradation in GBC-SD cells. Analysis of GSH revealed that PEITC induced a rapid GSH depletion, detectable after 1 hour of treatment (Figure 5A). As shown in Figure 5B–5C, PEITC increased GSSG levels and decreased GSH/GSSG ratio, which reflects the cellular redox state, after 6 hours of treatment. In comparison, CDDP only induced GSH and GSSG reduction with no apparent reduction in GSH/GSSG ratio (Figure 5D–5F). Therefore, these data suggest that PEITC can induce oxidative stress in GBC-SD cells. Coincidently, PEITC also facilitated Mcl-1 degradation after 6 hours of treatment (Figure 4B). Since there was no Mcl-1 degradation in the first few hours of PEITC incubation, it is likely that the depletion of GSH was a primary event that triggered a decrease in GSH/GSSG ratio and subsequent Mcl-1 degradation. In support of this hypothesis, supplementing cell culture medium with GSH precursor N-acetylcysteine (NAC) prevented PEITC-induced GSH depletion (Figure 5G), a decrease in GSH/GSSG ratio (Figure 5H), and Mcl-1 degradation (Figure 5I). Also, it significantly suppressed PEITC-CDDP-induced cell apoptosis (Figure 5J). Taken together, these data suggest that PEITC induces proteasomal degradation of Mcl-1 through depletion of GSH and a decrease in GSH/GSSG ratio.

PEITC induces proteasomal degradation of Mcl-1 by increasing the glutathionylated Mcl-1

Since Mcl-1 is a target of glutathionylation [7] and protein glutathionylation is greatly enhanced by decreased GSH/GSSG ratios that accompany cellular oxidative stress [18], we speculated that PEITC could increase the glutathionylated Mcl-1. Firstly, we found that endogenous Mcl-1 was partially glutathionylated under non-stressed conditions (Figure 6A). Furthermore, we found that DL-Dithiothreitol (DTT), a reducing agent, decreased the glutathionylated Mcl-1, and PEITC increased the glutathionylated Mcl-1 in a time-dependent manner (Figure 6B–6C).

Previous studies have demonstrated that glutathionylation of certain proteins may affect their functions and stability [18, 19]. We speculated that the glutathionylated Mcl-1 may be more susceptible to proteasomal degradation. Since only two cysteine residues, Cys16 and Cys 286, exist in the Mcl-1 protein, we investigated both these sites for potential glutathionylation. We used site-directed mutagenesis to convert these two cysteine residues Cys16 and Cys286, to serines (C16S, C286S, and C16S/C286S).

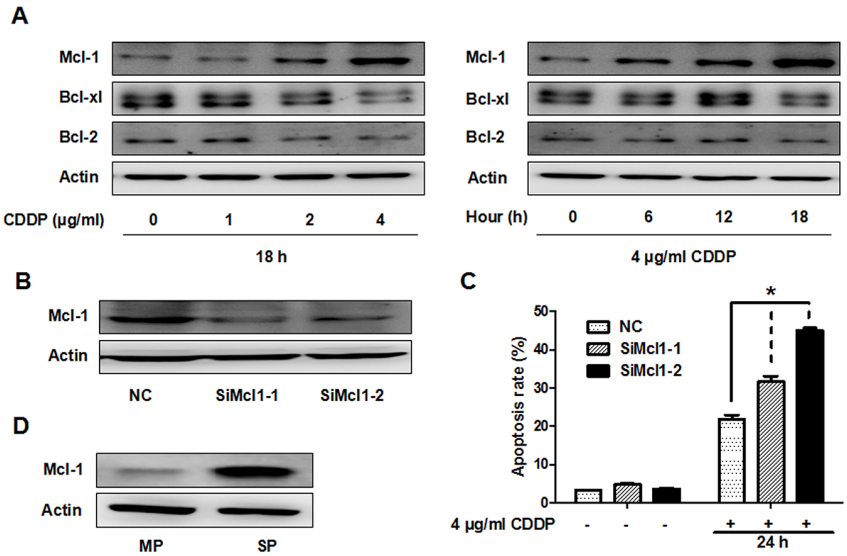

Figure 3: CDDP resistance is partially associated with Mcl-1 in GBC-SD and SP cells. (**A**) Immunoblot analysis of Mcl-1, Bcl-xl and Bcl-2 protein in GBC-SD cells. Left panel, cells were treated with the indicated concentrations of CDDP for 18 hrs. Right panel, cells were treated with 4 ug/ml CDDP and harvested at the indicated times. ß-Actin was used as a loading control. (**B**) Cells were transfected with non-specific siRNA (NC) or *Mcl-1* siRNA (SiMcl1) for 48 hrs and reduction in Mcl-1 was analysed by western blot (**C**) Apoptosis analysis using Annexin V/PI flow cytometry in GBC-SD cells transfected with *Mcl-1* siRNA after treatment with CDDP for 24 hrs. Data shown is average of three independent experiments. *$P < 0.05$. (**D**) Immunoblot analysis of Mcl-1 protein level in SP and MP cells from GBC-SD cells.

By examining the glutathionylation of Flag-Mcl-1 wild type (WT) and mutants, we found that the C16S mutant was weakly glutathionylated and the C286S mutant was modestly glutathionylated (Figure 6D, lanes 3–4), whereas the double mutant was devoid of any glutathionylation (Figure 6D, lane 5). Taken together, these findings suggest that both the two cysteine residues of Mcl-1 are glutathionylation sites. Finally, cells expressing Flag-Mcl-1 WT and C16S/C286S mutant were treated with PEITC for 24 hrs, and their protein levels were determined. Flag-Mcl-1 WT protein was decreased in PEITC-treated cells, while the C16S/C286S mutant was not sensitive to PEITC-mediated degradation (Figure 6E), suggesting that PEITC induces glutathionylation-dependent degradation of Mcl-1.

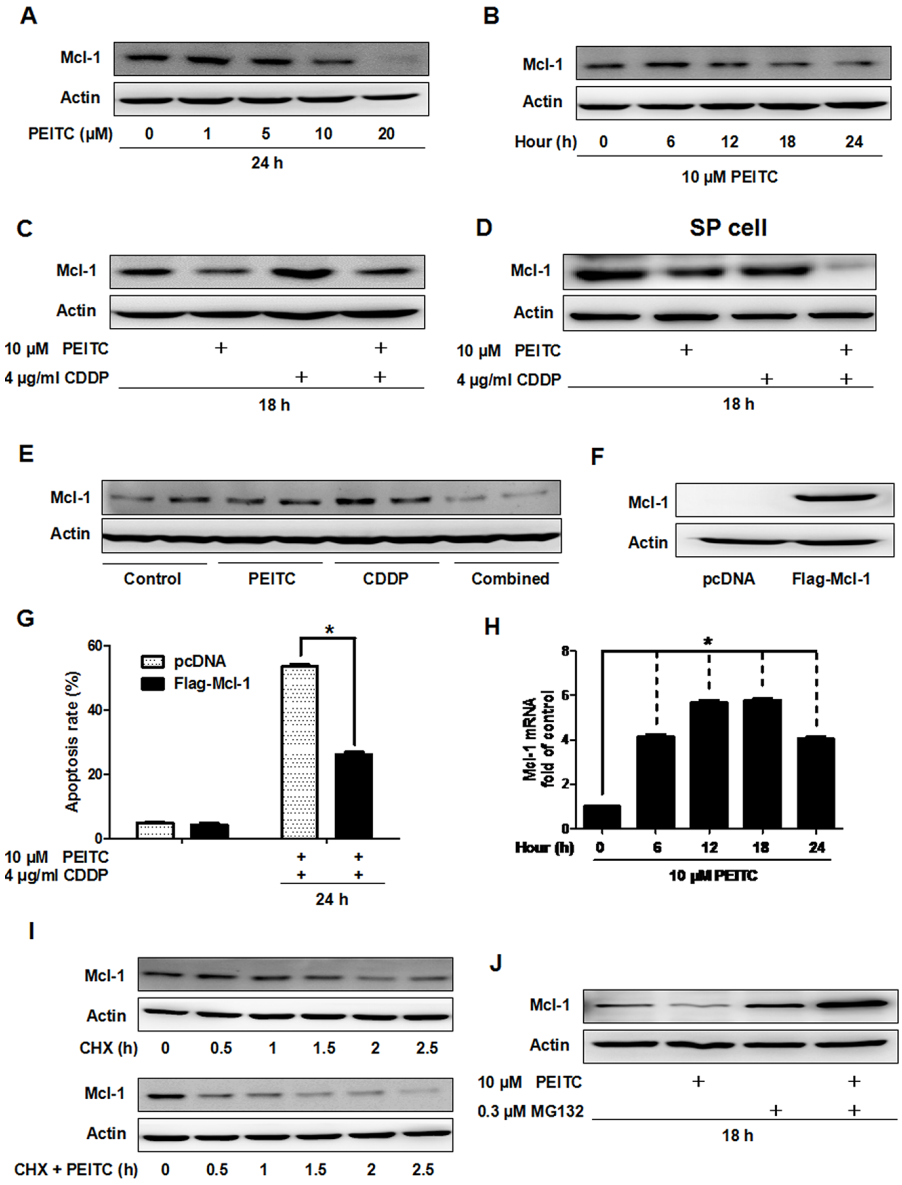

Figure 4: PEITC induces proteasomal degradation of Mcl-1 *in vitro* and *in vivo*. (**A**) Immunoblot analysis of Mcl-1 in GBC-SD cells after treatment with the indicated concentrations of PEITC for 24 hrs. (**B**) Mcl-1 protein was analysed after treatement with 10 uM PEITC for various times (**C**) Mcl-1 protein level was analysed after treatment with PEITC, CDDP or PEITC-CDDP combination for 18 hrs. (**D**) SP cells from GBC-SD cells were treated with PEITC, CDDP or PEITC-CDDP combination for 18 hrs, and Mcl-1 protein level analyzed. (**E**) Immunoblot analysis of Mcl-1 protein in tumor tissue extracts from control group; PEITC group; CDDP group; and PEITC-CDDP combination group. Tumor tissues were from two mice of each group. (**F**) Mcl-1 protein level was determined by western blot after transfection with vector or *Mcl-1* plasmid for 48 hrs. (**G**) Apoptosis analysis using Annexin V/PI flow cytometry in GBC-SD cells transfected with *Mcl-1* plasmid after treatment with PEITC-CDDP combination for 24 hrs. (**H**) Time course analysis of *Mcl-1* mRNA by 10 uM PEITC in GBC-SD cells, detected by quantitative real time PCR analysis. Data shown is average of three independent experiments. *$P < 0.05$. (**I**) Immunoblot analysis of Mcl-1 protein in GBC-SD cells. Cells were treated with 20 ug/ml cycloheximide for the indicated times or were pretreated with 10 uM PEITC for 6 hrs before exposure to 20 ug/ml cycloheximide. (**J**) Mcl-1 protein was analysed in cells treated with PEITC, MG132 or PEITC-MG132 combination for 18 hrs. ß-Actin was used as a loading control. CHX: cycloheximide.

DISCUSSION

CDDP-based chemotherapy is an important treatment regimen used in the clinical management of BTC, but the mechanisms for CDDP resistance are not entirely clear. Cancer cells may become resistant to platinum-based drugs through multiple mechanisms, such as an increased ability to repair DNA damage caused by platinum, neutralization of platinum toxicity, blocking platinum entry into the nucleus, an increase in drug export and so on [11, 20–24]. In addition, cisplatin resistance can develop through an increased ability to avoid drug-induced cell damage, cell shrinkage and hence initiation of apoptosis [25]. Apoptosis is regulated in part by the Bcl-2 family of proteins which consists of both pro-apoptotic and anti-apoptotic proteins [26]. Among all the anti-apoptotic Bcl-2 family members, Mcl-1 functions as a major survival factor, particularly in solid cancers [27].

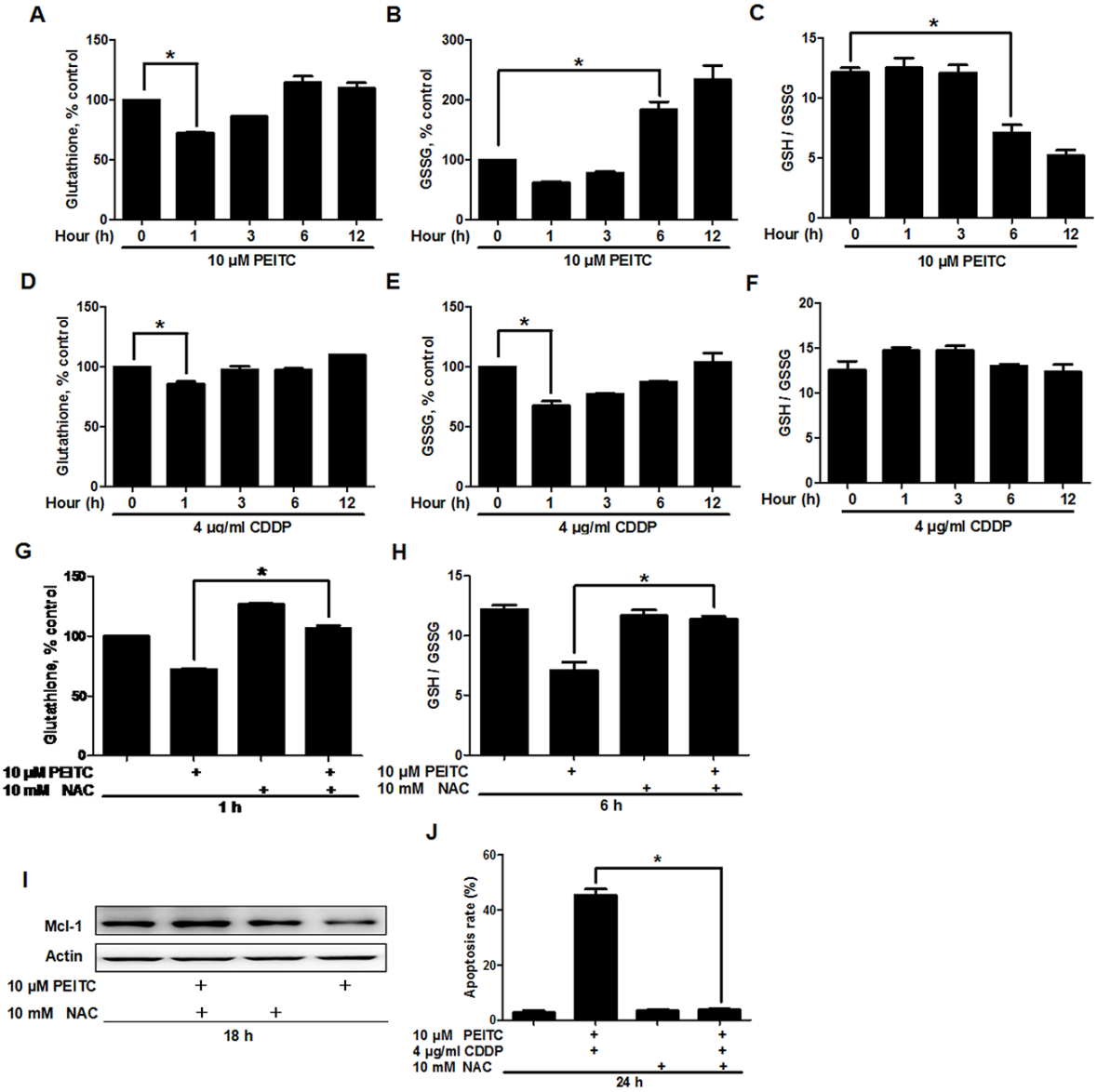

Figure 5: PEITC depletes GSH and decreases GSH/GSSG ratio. Analysis of (**A**) GSH levels, (**B**) GSSG levels and (**C**) GSH/GSSG ratio in GBC-SD cells, after treatment with 10 uM PEITC for the indicated times. Analysis of (**D**) GSH levels, (**E**) GSSG levels and (**F**) GSH/GSSG ratio in GBC-SD cells treated with 4 ug/ml CDDP for the indicated times. (**G**) Effect of NAC on PEITC-induced GSH depletion. GBC-SD cells were preincubated with 10 mM NAC for 12 hrs before treatment with 10 uM PEITC for 1 hour. (**H**) Effect of NAC on PEITC-induced decrease in GSH/GSSG ratio. GBC-SD cells were preincubated with 10 mM NAC for 12 hrs before treatment with 10 uM PEITC for 6 hrs. (**I**) Effect of NAC on PEITC-induced Mcl-1 degradation. GBC-SD cells were preincubated with 10 mM NAC for 12 hrs before treatment with 10 uM PEITC for 18 hrs. Mcl-1 protein was detected by western blot and ß-Actin was used as a loading control. (**J**) Effect of NAC on PEITC-CDDP-induced cell apoptosis. GBC-SD cells were preincubated with 10 mM NAC for 12 hrs before treatment with PEITC-CDDP combination for 24 hrs. Cell apoptosis was detected by Annexin V/PI assays. Data shown is average of three independent experiments. *$P < 0.05$.

Despite the confirmed importance of Mcl-1 in several cancers, the role of Mcl-1 in BTC survival has yet to be explored. In this study, we provided evidence that the effectiveness of CDDP is partially dependent on the Mcl-1 expression. Previously we found that Mcl-1 expression is increased in gallbladder carcinoma tissues [28]. Therefore investigation into new therapeutic strategies targeting Mcl-1 could prove crucial in treating BTC.

Several studies have discovered that PEITC is able to enhance the cytotoxic effect of CDDP in cancer cells [10, 11]. But the synergistic effect of PEITC and CDDP in BTC cells remains unknown. Moreover, little information is available concerning the functional role of Mcl-1 in mediating PEITC-induced chemosensitization. In this study, we found that PEITC can significantly enhance CDDP-induced apoptosis by degrading Mcl-1 in BTC

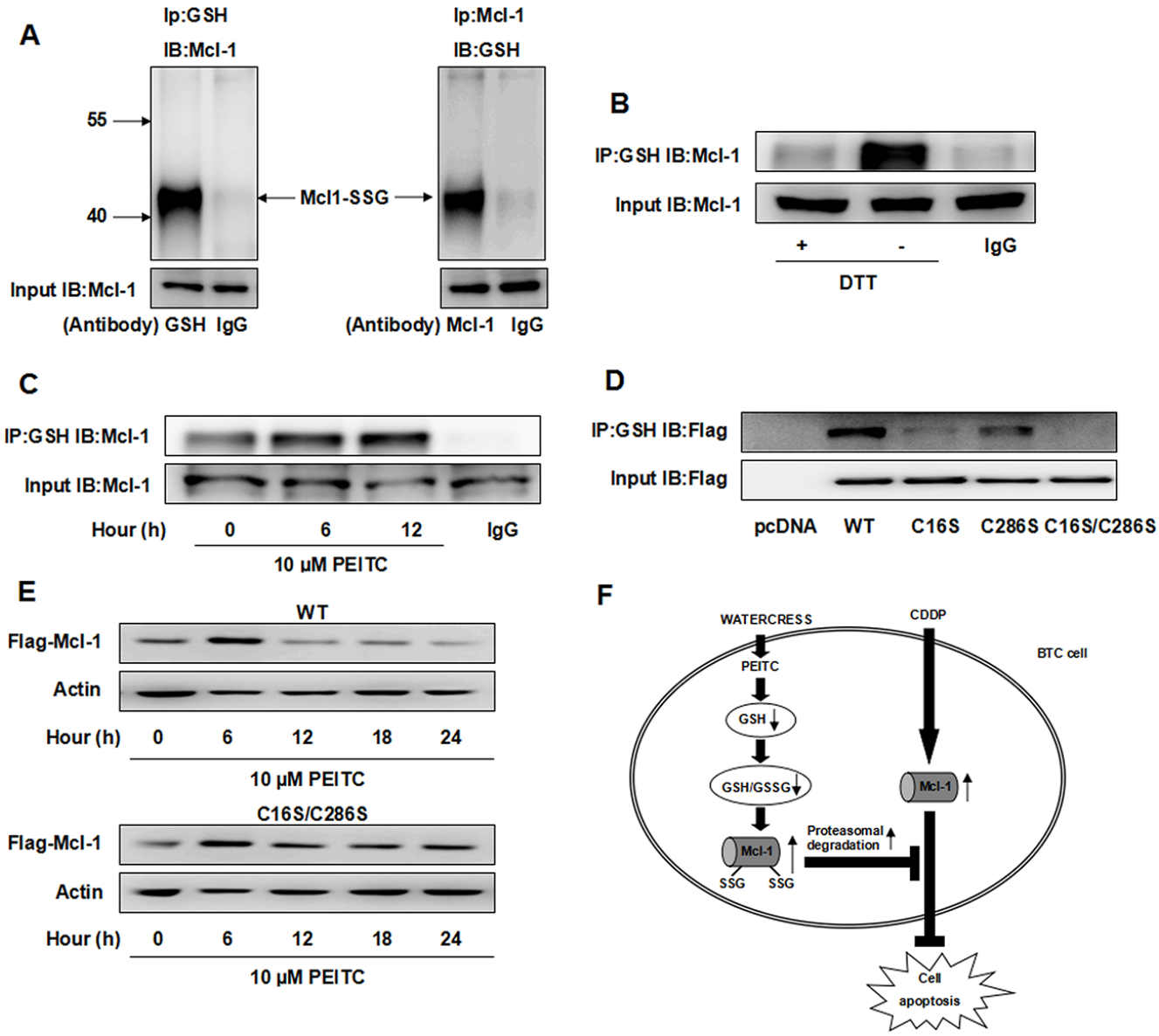

Figure 6: PEITC increases the glutathionylated Mcl-1 and induces glutathionylation-dependent degradation of Mcl-1. (**A**) Reciprocal IP/western analysis. Extracts from GBC-SD cells were immunoprecipitated using antibodies specific for Mcl-1 or GSH and the co-eluted proteins were detected by western blot with antibodies specific to GSH or Mcl-1. (**B**) GBC-SD cell lysate was treated with 10 mM DTT for 10 min before IP and the level of glutathionylated Mcl-1 was detected. (**C**) GBC-SD cells were treated with 10 uM PEITC and harvested at the indicated times and the level of glutathionylated Mcl-1 was detected by IP. (**D**) After transfection with Flag-Mcl-1 WT, C16S, C286S or C16S/C286S mutant for 48 hrs, level of glutathionylated Flag-Mcl-1 was detected by IP. (**E**) After transfection with Flag-Mcl-1 WT or C16S/C286S mutant for 48 hrs, GBC-SD cells were treated with 10 uM PEITC and harvested at the indicated times. Flag-Mcl-1 expression was detected by western blot and ß-Actin was used as a loading control. (**F**) Proposed model of combination effect of PEITC and CDDP in BTC cells. CDDP can increase Mcl-1 protein level, which is partially responsible for CDDP resistance. PEITC can deplete GSH, decrease GSH/GSSG ratio and increase the glutathionylated Mcl-1, making Mcl-1 more susceptible to proteasomal degradation. Mcl1-SSG: the glutathionylated Mcl-1. IB: immunoblot.

cells. Under mild oxidative stress, protein glutathionylation regulates functions of multiple proteins [18, 19]. Here, our results clearly show that Mcl-1 is glutathionylated in GBC-SD cells and a sub-toxic concentration of PEITC increases the glutathionylated Mcl-1, followed by rapid proteasomal degradation. Whereas, Trachootham et al. showed that PEITC renders Mcl-1 more susceptible to cleavage by caspase-3 by de-glutathionylation of Mcl-1 in chronic lymphocytic leukemia cells [7]. We speculate that the discrepancy is due to different cell lines and different concentration of PEITC in two studies. PEITC may have different effect on pathways existing in different cells, and may impact degradation pathway of Mcl-1. By mutagenesis, we identified and confirmed that both Cys16 and Cys286 are glutathionylation sites on human Mcl-1 protein. Although there was a consistent correlation between glutathionylation levels and proteasomal degradation of Mcl-1, the double mutant could not be glutathionylated (Figure 6D) and only partially prevented proteasomal degradation (Figure 6E). This suggests that PEITC-mediated degradation of Mcl-1 may involve not only glutathionylation-dependent degradation but also other mechanisms. It would be interesting to examine other mechanisms of PEITC-mediated degradation of Mcl-1.

Conventional chemotherapies for cancer cells are believed to mainly eliminate the majority of differentiated cancer cells but spare cancer stem cells, which are thought to be associated with recurrence [29–31]. In the present study, we used SP cells from GBC-SD cells as a model of cancer stem-like cells and found that SP cells have higher Mcl-1 expression, consistent with a recent report [32]. Moreover, PEITC enhanced the efficacy of CDDP by degrading Mcl-1 in SP cells, which implies that PETIC is a promising chemotherapy-sensitizing agent targeting cancer stem cells.

Previous studies showed that eating watercress can significantly increase the blood level of PEITC in humans [9] and an oral dose of 40 mg PEITC can result in a plasma concentration in the micromolar range within 3 to 8 hrs [33]. Thus, the effective concentration of PEITC appears to be achievable by oral supplementation in humans. Furthermore, a Phase I trial showed that at lower doses (40 and 80 mg daily for 30 days) PEITC was well-tolerated, and patients that consumed high doses of PEITC (120 and 160 mg daily for 30 days) showed only minor toxicity with low-grade diarrhea [5]. The fact that PEITC is found in our normal diets combined with its relatively low toxicity in humans provides strong support for a clinical investigation of PEITC-CDDP co-treatment in BTC.

In conclusion, CDDP resistance is partially associated with Mcl-1 in BTC cells and PEITC can enhance CDDP-induced apoptosis via glutathionylation-dependent degradation of Mcl-1. These results not only identify a novel mechanism of PEITC-enhanced chemosensitivity of BTC cells to CDDP, but also provide support that dietary intake of watercress may help reverse CDDP resistance in BTC patients.

MATERIALS AND METHODS

Cell culture and reagents

The human gallbladder cancer (GBC-SD) and human cholangiocarcinoma (RBE) cell lines were obtained from Cell Bank, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. GBC-SD and RBE cells were maintained in RPMI 1640 (Hyclone) supplemented with 10% fetal bovine serum (Hyclone). Cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C.

PEITC, NAC, Hoechst 33342, verapamil, DTT and cycloheximide were purchased from Sigma. MG132 was purchased from Calbiochem and CDDP was obtained from Qilu Pharmaceutical Co., Ltd (Jinan, China). PEITC was dissolved in dimethyl sulfoxide (DMSO) and was freshly diluted in culture media before use in experiments.

SP cell sorting from GBC-SD cells

SP analysis was performed as previously described [34]. Briefly, cells were trypsinized and resuspended in ice-cold Hank's balanced salt solution (Invitrogen). Hoechst 33342 was added at a final concentration of 5 mg/ml in the presence or absence of 50 mg/ml verapami. After incubating at 37°C for 90 min, the cells were analyzed by flow cytometry.

Cell viability, apoptosis analysis, CI and DRI

Cell viability was assayed using the MTT assay (Sigma) as previously described [35]. Cell apoptosis was assessed using an Annexin V-FITC/ PI kit (BD Pharmingen) and analyzed by flow cytometry on FACS Calibur (Becton Dickson) [36]. Cells that were positively stained by Annexin V-FITC only (early apoptosis) and positive for both Annexin V-FITC and PI (late apoptosis) were quantitated and both subpopulations were considered as overall apoptotic cells.

CI and DRI values of PEITC-CDDP co-treatment were determined using the CalcuSyn software using non-constant ratio combination design as previously described [37, 38].

Reverse transcription and real-time PCR

Reverse transcription and quantitative real-time PCR were carried out as previously described [39]. Primers sequences used in PCR analysis were as follows: *Mcl-1* forward 5′-TCCAAGGCATGCTTCGGA-3′ and reverse 5′-GGCACCAAAAGAAATGAGAGTCAC-3′; *GAPDH* forward 5′-GAAGGTGAAGGTCGGAGTC-3′ and reverse 5′-GAAGATGGTGATGGGATTTC-3′. Human *GADPH* mRNA served as an endogenous control for normalization.

GSH/GSSG ratio assay

GSH is a tripeptide with a free thiol group and function as a major antioxidant in cells. GSH/GSSG ratio reflects the cellular redox state. The total GSH and GSSG were determined by colorimetric microplate assay kit (Beyotime, China) as previously described [40, 41]. GSH/GSSG ratio was obtained {ratio = (Total GSH – 2GSSG)/GSSG}.

Mcl-1 siRNA and mutant transfection

Two specific siRNA oligonucleotides complementary to the *Mcl-1* mRNA sequence were transiently transfected into GBC-SD cells using Lipofectamine 2000 (Invitrogen) as previously described [42, 43]. A non-specific siRNA was also transfected as a mock control. After 48 hrs, GBC-SD cells were either lysed for western blot analysis or were exposed to CDDP for an additional 24 hrs and analysed for apoptosis using the methods described above. The siRNA-1 sequences complimentary for *Mc1–1* mRNA were 5′ GUAUCACAGACGUUCUCGUdTdT 3′ and 3′ dTdTCAU AGUGUCUGCAAGAGCA 5′. The siRNA-2 sequences complimentary for *Mcl-1* mRNA were 5′ GGACUUUU AGAUUUAGUGAdTdT 3′ and 3′ dTdTCCUGAAAAUC UAAAUCACU 5′.

Site-directed mutagenesis of the *Mcl-1* gene was performed using the Quick Change Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) using the pcDNA3.1-Flag-Mcl-1 plasmid as the template. Cells were transiently transfected with Flag-Mcl-1 WT and mutants using the above-mentioned Lipofectamine method. Nonrelevant plasmid pcDNA3.1 was used as the transfection control. After 48 hrs, GBC-SD cells were lysed for immunoprecipitation (IP) or treated with PEITC for an additional 24 hrs and analysed by western blot. The anti-Flag monoclonal antibody was purchased from Sigma.

Assays of Mcl-1 expression and glutathionylation

Mcl-1, Bcl-xl and Bcl-2 protein levels were determined by western blot as previously described [44]. Cellular glutathionylation of Mcl-1 was determined by IP with an anti-GSH antibody under non-reducing conditions, followed by western blot analysis [7, 18] using an anti-Mcl-1 antibody. Antibodies to Bcl-2 and Bcl-xl were purchased from Santa Cruz. Antibodies to GSH and Mcl-1 were purchased from Abcam.

In vivo study in tumor-bearing mice

All animal experiments were done in accordance with institutional guidelines for animal welfare. GBC-SD cells were harvested, washed, and resuspended in serum-free RPMI 1640 and then injected subcutaneously into 6-week old BALB/c-nu/nu mice (n = 28 mice, purchased from Shanghai Experimental Animal Center). Tumor volumes were measured twice a week using a calliper, and were calculated using the formula $V = \pi/6 \times \text{length} \times \text{width}^2$ [34, 45]. When the tumor size was approximately 50 mm^3, the mice were sorted into four equal groups (n = 7 mice per group). The tumor-bearing mice were intra-peritoneally administered with physiological saline as a control, PEITC (25 mg/kg), CDDP (2.5 mg/kg) or PEITC/CDDP twice a week. Tumor volumes were measured twice a week with a calliper and body weights were also recorded. The mice were sacrificed after 10 days, and body weight and tumor weight were measured.

Statistical analysis

Data were shown as mean value ± SD. SPSS17.0 software was used for statistical analysis. Analysis of variance was applied for comparison of the means of two or multiple groups. A value of $P < 0.05$ was considered significant.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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Clinical Trial of 2-Phenethyl Isothiocyanate as an Inhibitor of Metabolic Activation of a Tobacco-Specific Lung Carcinogen in Cigarette Smokers

Jian-Min Yuan[1,2], **Irina Stepanov**[3], **Sharon E. Murphy**[3,4], **Renwei Wang**[1], **Sharon Allen**[3], **Joni Jensen**[3], **Lori Strayer**[3], **Jennifer Adams-Haduch**[1], **Pramod Upadhyaya**[3], **Chap Le**[3], **Mindy S. Kurzer**[5], **Heather H. Nelson**[3,6], **Mimi C. Yu**[7], **Dorothy Hatsukami**[3], and **Stephen S. Hecht**[3]

[1]Division of Cancer Control and Population Sciences, University of Pittsburgh Cancer Institute, Pittsburgh, PA 15232

[2]Department of Epidemiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, PA 152323

[3]Masonic Cancer Center, University of Minnesota, Minneapolis MN 55455

[4]Department of Biochemistry, Molecular Biology and BioPhysics, University of Minnesota, Minneapolis, MN 55455

[5]Department of Food Science and Nutrition, University of Minnesota, St. Paul, MN 55455

[6]Division of Epidemiology and Community Health, School of Public Health, University of Minnesota, Minneapolis, MN 55455

[7]Department of Preventive Medicine, University of Southern California Keck School of Medicine, Los Angeles, CA 90089; Retired

Abstract

2-Phenethyl isothiocyanate (PEITC), a natural product found as a conjugate in watercress and other cruciferous vegetables, is an inhibitor of the metabolic activation and lung carcinogenicity of the tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in F344 rats and A/J mice. We carried out a clinical trial to determine whether PEITC also inhibits the metabolic activation of NNK in smokers. Cigarette smokers were recruited and asked to smoke cigarettes containing deuterium labelled [pyridine-D_4]NNK for an acclimation period of at least one week. Then subjects were randomly assigned to one of two arms: PEITC followed by placebo, or placebo followed by PEITC. During the one-week treatment period, each subject took PEITC (10 mg in 1 ml of olive oil, 4 times per day). There was a one week washout period between the PEITC and placebo periods. The NNK metabolic activation ratio: [pyridine-D_4]hydroxy acid/total [pyridine-D_4]NNAL was measured in urine samples to test the hypothesis that PEITC treatment modified NNK metabolism. Eighty-two smokers completed the study and were included in the

*To whom correspondence should be addressed: Masonic Cancer Center, University of Minnesota, 2231 6th Street SE - 2-148 CCRB, Minneapolis, MN 55455, USA. phone: (612) 624-7604, fax: (612) 624-3869, hecht002@umn.edu.

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analysis. Overall, the NNK metabolic activation ratio was reduced by 7.7% with PEITC treatment (P = 0.023). The results of this trial, while modest in effect size, provide a basis for further investigation of PEITC as an inhibitor of lung carcinogenesis by NNK in smokers.

Keywords

2-phenethyl isothiocyanate; PEITC; clinical trial; tobacco-specific lung carcinogen; chemoprevention

Introduction

Lung cancer, a devastating disease with a 5 year survival rate of less than 20%, killed 1,589,800 people in the world in 2012, about 4400 each day (1). Cigarette smoking accounts for 80% of this death toll in males and 50% in females (1). In the United States, cigarette smoking causes about 90% of all lung cancer deaths, which totaled 159,260 in 2014 (2). Eliminating or decreasing cigarette smoking are clearly the best approaches to save millions of lives; this has been partially successful in the United States, where the decrease in smoking prevalence is associated with a decrease in lung cancer (3). But this approach is limited because most cigarette smokers are addicted to nicotine and cannot quit their habit. About 70% of smokers try quitting each year, but less than 5% succeed (4). Even the best smoking cessation programs have six month success rates of only about 25% (5).

Chemoprevention is an alternate approach to decrease the risk for lung cancer in addicted smokers (6). In chemoprevention, one is treating the carcinogenic process rather than attempting to eradicate an established tumor. If carcinogenesis can be halted or even slowed, the chances of averting fatal lung cancer are increased. Tobacco smoke carcinogens are prime targets for chemoprevention of lung cancer; if their effects can be eliminated or decreased, the probability of developing lung cancer would also be decreased (6).

It has been 30 years since Chung et al first showed that phenethyl isothiocyanate (PEITC), which is found in substantial quantities as the conjugate gluconasturtiin in watercress and several other cruciferous vegetables, inhibited an obligatory step in the metabolic activation of the tobacco-specific lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK, Figure S1) (7). NNK, which selectively induces lung tumors in rats, mice, and hamsters, is considered to be one of the most important carcinogens in tobacco smoke, and has been evaluated by the International Agency for Research on Cancer, along with the related carcinogen *N*′-nitrosonornicotine (NNN), as “carcinogenic to humans.” (8,9) Thus, decreasing the carcinogenicity of NNK by inhibiting its metabolic activation is one logical approach to chemoprevention of lung cancer in smokers.

Following the observation by Chung et al, multiple studies demonstrated that PEITC as well as its major metabolite *N*-acetyl-*S*-(*N*-2-phenethylthiocarbamoyl)-L-cysteine (PEITC-NAC) significantly inhibited lung tumorigenesis by NNK in mice and rats (10–17). Studies in the A/J mouse noted up to complete inhibition of lung tumor induction by NNK (for example, from 10.7 lung tumors per mouse to 0.3 lung tumors per mouse) when PEITC was given prior to NNK (11), but not when it was administered after NNK, indicating that its main

effect was on metabolism or DNA binding of NNK. Similarly, three long term bioassays in F-344 rats, in which PEITC was added to the diet, clearly demonstrated significant inhibition of lung tumor induction by NNK (10,12,13). One study noted complete inhibition of lung tumor development (12).

Multiple studies have clearly demonstrated that the major mechanism by which PEITC inhibits carcinogenesis by NNK is inhibition of its metabolic activation by cytochrome P450 enzymes. This is the crucial step that produces highly reactive methylating and pyridyloxobutylating species that react with DNA to form a variety of mutagenic DNA adducts which are critical in lung carcinogenesis by NNK (Figure S1 (8,18). These studies, which have been carried out under a wide variety of conditions both in vitro and in vivo, with protocols varying from acute to chronic, and in some cases mimicking or coinciding with conditions used in the carcinogenicity studies, consistently demonstrate that PEITC decreases the formation of critical reactive metabolites of NNK, resulting in lower levels of DNA adducts in the lung, lower levels of hemoglobin adducts, and decreases in other endpoints, all reflecting inhibition of NNK metabolic activation (7,10–12,15,19–25). Nearly all of this can be traced to the inhibitory effects of PEITC on cytochrome P450 enzymes including human P450s 2A13, 2A6, 1A2, and 2B6, which are catalysts of NNK bioactivation (20,23,24,26–33). Consistent with these observations, we observed a significant effect of watercress consumption, as a source of PEITC, on NNK metabolism in smokers in a small clinical study (34). Excretion of the NNK metabolites NNAL plus NNAL-glucuronides increased upon watercress consumption, which would be expected upon inhibition of NNK metabolic activation (Figure S1). However, watercress consumption did not inhibit metabolism of coumarin or nicotine, both catalyzed by P450 2A6 (35,36).

Another aspect of chemoprevention by isothiocyanates involves the glutathione-*S*-transferase enzymes (GSTs) which catalyze the conjugation of isothiocyanates such as PEITC with glutathione, leading to the excretion in urine of PEITC-NAC. It has been hypothesized based on epidemiologic studies that individuals with *GSTM1* or *GSTT1* null-null genotypes would be protected from cancer more effectively by isothiocyanates than those with competent GST enzymes, because the isothiocyanates would be less effectively metabolized and excreted as their conjugates. Some support for this hypothesis has been obtained (37), but one study demonstrated no effect of *GSTT1* or *GSTM1* status on isothiocyanate excretion after consumption of watercress juice (38,39).

Thus, convincing data in the literature leave no doubt that PEITC can inhibit the metabolism and carcinogenicity of NNK in the rat and mouse lung. The primary aim of the randomized trial described here was to determine whether PEITC would also inhibit the metabolism of NNK in cigarette smokers. We applied our previously developed unique methodology based on the use of special cigarettes containing [pyridine-D_4]NNK to allow us to quantify the extent of its metabolic activation to α-hydroxy metabolites which ultimately produce DNA adducts (Figure S1) (40). Formation of these metabolites was assessed by quantifying levels of [pyridine-D_4]hydroxy acid, as opposed to the relatively large amounts of unlabeled urinary hydroxy acid produced as a minor urinary metabolite of nicotine in all smokers. To account for possible differences in the extent or topography of smoking of these cigarettes containing [pyridine-D_4]NNK during the trial, we quantified the ratio [pyridine-D_4]hydroxy

acid:total [pyridine-D_4]NNAL, where total [pyridine-D_4]NNAL is the sum of [pyridine-D_4]NNAL and its glucuronides (Figure S1). This ratio was hypothesized to decrease in subjects who took PEITC, due to the inhibition of cytochrome P450-catalyzed metabolic activation of [pyridine-D_4]NNK and [pyridine-D_4]NNAL (Figure S1). A secondary aim was to determine whether *GSTM1* and *GSTT1* genotype modified the impact of PEITC on NNK metabolism. This is the first clinical trial to assess the effects of pure PEITC on carcinogen metabolism in humans.

Materials and Methods

Drug Product

The use of PEITC and [pyridine-D_4]NNK in this study were approved by the U.S. Food and Drug Administration under IND 74,037. Bulk PEITC was purchased from Sigma-Aldrich Corp, St. Louis, MO, and purified under GMP conditions at the Molecular and Cellular Therapeutics Facility, University of Minnesota. Purification was carried out on a Teledyne ISCO 1500 g flash column attached to a Teledyne ISCO Automated Chromatograpy System Combiflash Companion XL. PEITC was eluted by sequential washing with hexane and 80% hexane/20% ethanol. Fractions corresponding to PEITC standard, as determined by thin layer chromatography, were combined and the solvents were removed under reduced pressure. The purity of the PEITC was 99.2%, as determined by HPLC with UV detection at 254 nm, ^{1}H-NMR, ^{13}C-NMR, and GC-MS. Each dose used in this study consisted of 10 mg PEITC dissolved in 1 ml of olive oil N.F. (Gallipot, Inc). This solution was placed in a plastic syringe which each subject used to squirt the material into his or her mouth, 4 times per day, once every 4 h. Placebo was 1 ml of olive oil in a plastic syringe, with no added PEITC.

[pyridine-D_4]4-Methylnitrosamino-1-(3-pyridyl)-1-butanone ([pyridine-D_4]NNK)

[pyridine-D_4]NNK was synthesized from [pyridine-D_4]ethyl nicotinate (Toronto Research Chemicals) essentially as described for NNK (41). Its purity was >99% as determined by ^{1}H-NMR, HPLC with UV detection, GC-MS, and direct inlet MS.

Cigarettes containing [pyridine-D_4]NNK

Marlboro Virginia Blend (king size, hard pack) cigarettes, which contained 0.21 – 0.37 μg NNK/g wet weight tobacco, were purchased on the open market and modified by adding 0.300 μg [pyridine-D_4]NNK to each cigarette. The addition of [pyridine-D_4]NNK to the cigarettes was carried out with a specially designed microsyringe applicator system as previously described (40). The prepared cigarettes were conditioned at 25 °C and 60% relative humidity for 2 days, placed back in their original packs, 20 cigarettes per pack, and stored at 4 °C until being dispensed to study subjects. Twenty-five batches of these cigarettes were smoked under US Federal Trade Commission standard conditions; 10 batches were analyzed at the University of Minnesota, and contained 22.4 ± 2.1 ng/cigarette NNK and 21.5 ± 3.9 ng/cigarette [pyridine-D_4]NNK in their mainstream smoke; and 15 batches were analyzed at the Centers for Disease Control and Prevention, Atlanta, GA and contained 18.8 ± 3.7 ng/cigarette NNK and 19.2 ± 2.4 ng/cigarette [pyridine-D_4]NNK in their mainstream

smoke. The use of these cigarettes was approved by the FDA Center for Tobacco Products, under Protocol Number P00006.

Study Design

This study was approved by the Institutional Review Boards of the University of Minnesota and the University of Pittsburgh and conducted at the University of Minnesota. The study was a phase II, randomized, placebo-controlled, double-blind, crossover clinical trial. The eligibility criteria for the study were: 1) current smokers of 10–45 cigarettes/day who were 21 years or older; 2) in apparent good physical health with normal liver function and no unstable medical conditions such as seizures or cancer; 3) in stable and good mental health, i.e., currently not experiencing unstable or untreated psychiatric diagnosis including substance abuse during the past 6 months; 4) currently not using any other tobacco or nicotine-containing products other than cigarettes; 5) not taking any drugs known to be substrates of cytochrome P450 1A2, 2B6 and 2A13 that may influence NNK metabolism; and 6) for female subjects, not pregnant or nursing. An overview of the study design is presented in Figure S2.

The duration of the study was approximately 5 weeks. After a telephone screening interview for subjects who responded to our local advertisement of the study, we invited those who were potentially eligible for the study to our research clinic for an orientation session. At the initial clinic visit, informed consent was obtained and subjects were asked to complete a questionnaire that asked for histories of tobacco use, medical conditions and medication use. A blood sample was collected for hemogram and liver function test. This blood sample also was used to determine both *GSTM1* and *GSTT1* genotypes for potential eligible subjects in the first two years of recruitment. After this visit (Week 1), subjects who were confirmed to be eligible were asked to smoke study cigarettes containing [pyridine-D_4]NNK for at least one week (smoking adaptation) before the treatment began (Week 2). They were asked to smoke only these study cigarettes throughout the entire study period. Subjects were then randomly assigned to either the PEITC then placebo arm (PEITC-placebo group), or the placebo then PEITC arm (placebo-PEITC group) of the trial. During the treatment period, each subject was asked to take PEITC (10 mg in 1 ml olive oil, 4 times/day, once every 4h, for five days; Week 3 or 5) or the placebo agent (olive oil), on the same schedule (Week 3 or 5). There was a one-week washout period between the PEITC and placebo treatments (Week 4). Subjects were asked to avoid eating cruciferous vegetables, as instructed by a dietitian, throughout the study. Study participants were also asked to record any food and beverages consumed over 24h for 3 days (3^{rd}, 4^{th} and 5^{th}) of each of the two treatment periods. The 24h food diaries were checked on a daily basis at the clinic visits during two treatment periods to confirm that the study participants did not consume any cruciferous vegetables during the past 24h.

The 24 h urine samples were collected at the following time points: end of the smoking adaptation period (week 2), three days (3^{rd}, 4^{th}, and 5^{th} day) in each of the two treatment periods (weeks 3 and 5), and at the end of the washout period (week 4). Spot urine samples were collected on the 2^{nd} day of each treatment period. Blood and buccal cell samples were

collected at the following time points: baseline, end of the smoking adaptation period, each of the two treatment periods, and the washout period.

Analyses of Urinary Biomarkers

Total [pyridine-D_4]NNAL and total NNAL were measured as described (40). Urine samples were treated with β-glucuronidase to release NNAL from its *N*- and *O*-glucuronides, and further purified and analyzed by LC-ESI-MS/MS. Free [pyridine-D_4]NNAL and free NNAL were analyzed by the same method, but the urine samples were not treated with β-glucuronidase prior to their purification. Analysis of [pyridine-D_4]hydroxy acid was performed by our recently developed methodology as described (42). Nicotine metabolites in urine including nicotine, cotinine and 3′-hydroxycotinine were analyzed as described (43). We used previously described methods, HPLC with UV-detection, for quantification of PEITC-NAC (44) and total isothiocyanates (45).

Endpoint Measures

The primary endpoint in this study was the ratio of urinary [pyridine-D_4]hydroxy acid: total [pyridine-D_4]NNAL. The rationale for use of this ratio is that it is not expected to be influenced by the number of cigarettes smoked per day by each subject or by smoking topography. As shown in Figure S1, [pyridine-D_4]hydroxy acid is a urinary metabolite resulting from metabolic activation of [pyridine-D_4]NNK. The other major urinary metabolite that results from this pathway is [pyridine-D_4]keto acid. In the analysis of [pyridine-D_4]hydroxy acid, the urine is treated with $NaBH_4$, which converts all [pyridine-D_4]keto acid to [pyridine-D_4]hydroxy acid. Total [pyridine-D_4]NNAL is the sum of the free metabolite and its glucuronides. As shown in Figure S1, total NNAL is produced by a pathway of NNK metabolism that is catalyzed mainly by carbonyl reductase enzymes, and total NNAL is considered to be an excellent biomarker of NNK exposure (46). These considerations lead to the conclusion that the ratio [pyridine-D_4]hydroxy acid: total [pyridine-D_4]NNAL is a biomarker of NNK/NNAL metabolic activation, which we hypothesized would be inhibited by PEITC. The deuterated compounds are necessary because hydroxy acid is a minor metabolite of nicotine, thus being present in smokers' urine at more than 1000 times the amounts which could be generated in metabolism of NNK (47).

Secondary endpoints were urinary metabolites of NNK (total and free NNAL) and nicotine (total cotinine, 3′-hydroxycotinine, and their glucuronides). We calculated the total 3′-hydroxycotinine:total cotinine ratio, a measure of CYP2A6 activity, at the end of smoking adaptation period.

Statistical Analyses

All urinary biomarkers were expressed per mg of urinary creatinine to account for varying water content of individual urine samples. Given the markedly skewed distributions of the endpoint measures, formal statistical testing was performed on logarithmically (natural log) transformed values, and geometric means are presented.

The two-group *t*-test (for continuous variables) or chi-square statistics (for discrete or nominal variables) were used to compare the differences between the two treatment

sequence assignments. The analysis of variance (ANOVA) method was used to examine the differences in urinary total ITC and PEITC-NAC among different *GSTM1/GSTT1* genotypes. Spearman correlation was used to evaluate the correlations among urinary biomarkers measured before the PEITC treatment (at visit 2).

This study was a randomized crossover trial, a type of longitudinal study in which participants were randomly assigned to receive a sequence of treatment of PEITC or placebo. We used the linear mixed model with random effect (48) to simultaneously examine the effect of treatment (PEITC versus placebo), study period (period 1 versus period 2), treatment sequence (the carryover effect) and their interaction on the endpoint measurements in all subjects as well as in subgroup analyses stratified by subjects' characteristics such as *GSTM1* and/or *GSTT1* genotypes, age, sex and smoking. A series of interaction terms between PEITC treatment and the baseline characteristics were included in the linear mixed models to evaluate the potential modifying effect of these factors on PEITC's effect on the primary outcome measure – the [pyridine-D_4]hydoxy acid:total [pyridine-D_4]NNAL ratio. Because the statistical analyses for all the primary and secondary outcomes were done on the log-transformed variables, the difference of log-transformed means after back-transformation is presented as the percentage change, the equivalents of the ratio of the least-squared means on the original scale.

Statistical analyses were carried out using SAS software version 9.3 (SAS Institute, Cary, NC). All *P*-values reported are two-sided, and those that were less than 0.05 were considered to be statistically significant.

Results

Basic Observations

Figure 1 summarizes subject flow through the study. We interviewed 589 subjects, of whom 311 were potentially eligible for the study. Those subjects were invited to come to our research clinic for further evaluation of their eligibility. Of the 205 who visited our research clinic and signed consent forms, 110 passed the physical examination and enrolled in the study. Of those enrolled, 86 completed the entire course of the study and 24 withdrew. In addition, 4 subjects had undetectable urinary total [pyridine-D_4]NNAL, indicating that they did not smoke the cigarettes containing [pyridine-D_4]NNK, and thus were excluded from all statistical analysis.

Among those who completed the study, the main adverse events during the PEITC treatment period were mild gastrointestinal (GI) disorders including dry mouth, taste alteration, stomach ache, belching, flatulence, and diarrhea; none of these were in the severe grade category.

Characteristics of the study subjects are summarized in Table 1. There were no significant differences in distributions of age, gender, race, level of education, smoking history (smoking intensity, duration, and age when beginning to smoke), and alcohol consumption between the PEITC-Placebo and Placebo-PEITC groups. Overall, the mean age (SD) was 41.0 (10.1) years. Among the 82 study participants, 46% were women, 67% were whites and

22% were African Americans. On average, study subjects smoked 19.1 (6.6) cigarettes per day and smoked cigarettes for 16.3 (9.8) years. Forty-seven percent had non-null and 12% null-null genotypes of both *GSTM1* and *GSTT1* genotypes.

At the end of the smoking adaption (pre-intervention) period, study participants smoked an average of 20.9 (7.8) [pyridine-D_4]NNK cigarettes per day. There was no statistical difference in mean [pyridine-D_4]NNK cigarettes per day between the treatment sequence assignments at pre-intervention (Table 1) and during the intervention period (Table S1). The number of [pyridine-D_4]NNK cigarettes smoked per day was comparable during study period 1 and period 2 (Table S1). At the pre-intervention stage, urinary levels of nicotine, NNK, and [pyridine-D_4]NNK metabolites except urinary total nicotine were comparable between smokers assigned to the PEITC-Placebo and Placebo-PEITC groups (Table 2).

Correlations among various parameters measured in this study are shown in Table S2. The number of [pyridine-D_4]NNK cigarettes per day, urinary levels of NNAL and [pyridine-D_4]NNAL, both total and free, and all nicotine metabolites were highly correlated ($P<0.001$); [pyridine-D_4]hydroxy acid was highly correlated with free and total [pyridine-D_4]NNAL and with free and total NNAL.

Primary Outcome Analyses

Urinary levels of both PEITC-NAC and total isothiocyanates (ITC) were significantly elevated in the PEITC treatment periods compared to the placebo periods among all subjects by a magnitude of more than 150 times (Table S3). On average, approximately 29% of the daily PEITC intended dose was excreted as PEITC-NAC in the urine (Table S3).

Intake of PEITC reduced the urinary ratio of [pyridine-D_4]hydroxy acid: total [pyridine-D_4]NNAL by 7.7% (95% CI: 1.2% to 13.8%; P = 0.023) (Table 3). This reduction was primarily due to decreased [pyridine-D_4]hydroxy acid, a biomarker of NNK metabolic activation, by PEITC. No statistically significant treatment sequence (i.e., carryover) effect was observed for urinary total [pyridine-D_4]NNAL, [pyridine-D_4]hydroxy acid or their ratio (Table S4). However, there was a statistically significant increase in urinary total [pyridine-D_4]NNAL and a significant decrease in the ratio of [pyridine-D_4]hydroxy acid: total [pyridine-D_4]NNAL during study period 2 compared with study period 1 (Table S4).

The potential influence of *GST* genotype on PEITC metabolism was examined and the results are shown Table S5. Individuals possessing GSTM1 or both GSTM1 and GSTT1 genes had lower urinary concentrations of PEITC-NAC and total ITC than their counterparts lacking the genes, respectively.

The potential influence of *GST* genotype on PEITC's effect on [pyridine-D_4]NNK metabolism was examined and the results are shown Table 4. PEITC inhibited [pyridine-D_4]NNK metabolism in subjects with the *GSTT1* null genotype (an 11.1% decrease) but in *GSTM1* non-nulls (an 8.8% decrease). The inhibitory effect of PEITC on [pyridine-D_4]NNK metabolism was strongest in subjects lacking both the *GSTM1* and *GSTT1* genes (a 15.6% decrease, P=0.039), followed by those possessing both genes (a 9.4% decrease, P=0.045), but there was no effect in those possessing either the *GSTM1* or the *GSTT1* gene. However,

the differences between these genotype groups were not statistically significant (all *P* values for interaction ≥ 0.436).

We also examined the effect of PEITC on the urinary [pyridine-D_4]hydroxy acid: total [pyridine-D_4]NNAL ratio stratified by age (<40, ≥40 years), sex, years of smoking, total nicotine equivalents, and total 3′-hydroxycotinine:total cotinine ratio. Stronger effects of PEITC intake on the reduction of urinary [pyridine-D_4]hydroxy acid: total [pyridine-D_4]NNAL ratio were observed for subjects who were 40 years or older, were women, and had lower total nicotine equivalents or higher CYP2A6 activity (Table 5). However, the differences between these groups were not statistically significant (all *P* values for interaction ≥ 0.245).

Secondary Analyses

We analyzed the urine of some subjects for [pyridine-D_4]4-hydroxy-1-(3-pyridyl)-1-butanone ([pyridine-D_4] keto alcohol) and [pyridine-D_4]4-hydroxy-1-(3-pyridyl)-1-butanol ([pyridine-D_4] diol), which are primary metabolites of NNK and NNAL formed by their metabolic activation pathways (Figure S1), but neither was detectable.

Supplementation with PEITC significantly increased urinary total NNAL and total nicotine, and increased the ratio of 3′-hydroxycotinine glucuronides to free 3′-hydroxycotinine (Table S6). When data were analyzed by *GSTM1* and *GSTT1* genotypes, the effect of PEITC supplementation on the increased ratio of 3′-hydroxycotinine glucuronides to free 3′-hydroxycotinine was seen mainly in subjects who possessed either the *GSTM1* (by 33.1%, 95% CI 6.8%–65.8%) or *GSTT1* gene (by 31.3%, 95% CI 6.0% – 62.75), and was especially elevated among those who had both *GSTM1* and *GSTT1* genes (43.6%, 95% CI 15.5%–78.7%). There were no statistically significant effects of PEITC on urinary excretion of free NNAL, total cotinine, total 3′-hydroxycotinine, or total nicotine equivalents; and the total 3′-hydroxycotinine:total cotinine ratio, the glucuronides:free NNAL ratio, the glucuronides:free [pyridine-D_4]NNAL ratio, or the glucuronides:free cotinine ratio (Table S6). *GSTM1* and/or *GSTT1* status did not modify the effects of PEITC on the urinary levels of these NNK and nicotine metabolites or their glucuronides/free ratios (data not shown).

Discussion

This is the first clinical trial of PEITC as an inhibitor of NNK metabolism in smokers. We used a unique design featuring cigarettes containing [pyridine-D_4]NNK to allow specific evaluation of the potential effect of PEITC on NNK metabolism without confounding by nicotine metabolites. We report a significant but modest 7.7% inhibitory effect of PEITC on the metabolic activation of NNK in our smokers. Although modest, these findings were consistent with our hypothesis based on extensive studies of PEITC effects on NNK metabolism and carcinogenicity in laboratory animals. Stronger effects of PEITC on NNK metabolism were observed in subjects with the null genotype of both *GSTM1* and *GSTT1* (reduced by 15.6%), as well as in women, subjects 40 years or older, and those with higher total 3′-hydroxycotinine :total cotinine ratio, a phenotypic measure of CYP2A6.

We also observed a statistically significant effect of PEITC on glucuronidation of 3′-hydroxycotinine (a 22.9% increase) as in our previous study with watercress (35). The magnitude of the effect of PEITC on 3′-hydroxycotinine glucuronidation was greater in individuals with at least one copy of *GSTM1* or *GSTT1*, especially in those possessing both *GSTM1* and *GSTT1* genes. These results suggest that PEITC may have a stronger effect on the induction of glucuronidation in individuals with the *GSTM1* and *GSTT1* genes, but as noted below, the effects of *GSTM1* and *GSTT1* status in this study were complex and are poorly understood.

Intake of PEITC borderline significantly increased urinary total nicotine (by 8.9%) and total nicotine equivalents (by 6.0%). This could be due to the bitter taste of PEITC delivered in olive oil by syringe. Subjects may have changed their smoking behavior to alleviate the unpleasant taste and smell of PEITC. However, we did not find that subjects consumed significantly more cigarettes in the PEITC treatment period than the placebo period. In our exploratory analysis (Table 5), a stronger inhibitory effect of PEITC on NNK metabolism was seen in smokers with lower total nicotine equivalents (a 10.7% decrease in [pyridine-D_4]hydroxy acid:total [pyridine-D_4]NNAL ratio) than higher total nicotine equivalents (a 6.8% decrease). However, the difference was not statistically significant ($P = 0.544$).

The daily dose of 40 mg PEITC was chosen based on the results of our study with watercress, in which subjects consumed a daily minimum amount of PEITC ranging from 19–38 mg for 3 days that effectively increased urinary total NNAL excretion (34). This dose did not show any toxic effects. In the Phase I study of PEITC, doses of up to 120 mg per day for 30 days were investigated. This highest dose caused some gastrointestinal distress and diarrhea, more than the 40 mg dose used here. While this 40 mg dose (0.5 mg/kg/day) is considerably less than the dose of PEITC used in our rat studies (20 mg/kg/day), so is the dose of NNK in our study subjects (0.000012 mg/kg/day), considerably less than that used in the rat studies (0.118 mg/kg/day). Thus, the PEITC:NNK ratio in our study subjects was about 42,000, which was about 250 times higher than the corresponding figure in the rat study (PEITC:NNK ratio = 170). PEITC is fat soluble. Thus, delivery of PEITC in olive oil avoids concerns about bioavailability; the phase 1 trial used the same form. The half-life of PEITC was 2.4h in the phase 1 study. Thus study subjects were asked to take PEITC (10 mg 4 times per day) to have a relatively stable internal dose of PEITC throughout the 24h per day.

Nevertheless, the dosing form of PEITC used in this study was not optimal and the dose may have been too low. The urinary excretion of PEITC equivalents (measured as PEITC-NAC) averaged 13.5 mg in 24 h in the study participants, which was approximately only one-third the amount of PEITC equivalents (36.6–37.7 mg) excreted as PEITC-NAC in the 24 h urine of 11 subjects who consumed six ounces of watercress per day (34). The lower excreted amount of PEITC-NAC in the present study suggests at least two possibilities – a low to moderate compliance among subjects who consumed a lower amount of PEITC than provided or retention of PEITC in the body that delays urinary excretion. Also, in the Phase I study with a daily dose of 120 mg PEITC, the maximum amount of PEITC-NAC in the urine was not reached until 2 weeks of daily dosing. This suggests that a longer dosing period and/or higher dose might have been more successful in our trial. Our study design

was a compromise that was partially influenced by practical considerations such as the available dosage form.

Following the initial observation by London and co-workers that individuals with deletions in *GSTM1* and *GSTT1* who consumed isothiocyanates were at decreased risk for lung cancer (38), many studies have examined this relationship. A review of this literature suggests that cruciferous vegetable intake may be weakly and inversely associated with lung cancer risk with the strongest protective association among those with homozygous deletion for both *GSTM1* and *GSTT1*, based on the hypothesis that these individuals would have more free isothiocyanate available (37). However, Dyba et al did not find any effect of *GSTM1* and *GSTT1* genotype on excretion of isothiocyanates after consumption of watercress juice, a rich source of PEITC (39). We also did not observe the predicted effect of glutathione *S*-transferase polymorphisms on PEITC metabolism. We saw a *increases* in urinary PEITC-NAC in the subjects who were null for *GSTM1* and *GSTT1* compared to those with the non-null genotypes. Similarly, subjects with *GSTM1 null* genotype had significantly *more* total isothiocyanates in their urine than those with the non-null genotype. These data suggest that GSTM1 and GSTT1 did not enhance the urinary excretion of ITC or PEITC. Based on our original hypothesis, we would have expected a stronger inhibitory effect of PEITC on NNK metabolic activation in those individuals with *GST* null genotypes. However, the present study did not show consistent results. PEITC had a stronger inhibitory effect in subjects with the null genotype of *GSTT1* but not *GSTM1*. Furthermore, the inhibitory effect of PEITC on NNK metabolism was comparable in subjects who lacked both or possessed both genes. These data suggest a complex role of *GSTM1* and *GSTT1* in modifying the potential inhibitory effect of PEITC on NNK metabolism. It should also be noted that *GSTP1*, which has polymorphic forms but has not been shown to exist as a null, is also an excellent catalyst of PEITC metabolism (49), and that PEITC reacts easily with glutathione without catalysis. Further studies are required to elucidate the mechanisms by which *GST* null status affects isothiocyanate metabolism and lung cancer susceptibility.

The present study is larger and better controlled, both with respect to evaluation of NNK metabolism and specificity of the chemopreventive agent compared to our earlier study using watercress. Nevertheless, watercress consumption is an attractive way to deliver PEITC because of the relatively high concentrations of the PEITC precursor gluconasturtiin in this vegetable, which can be consumed in a more pleasing way than the dosing form used here, and without any significant concerns about toxicity. It will be important to build on the current trial results using watercress as the potentially protective agent.

The analysis of [pyridine-D_4]hydroxy acid in urine is difficult because this highly polar metabolite is found in relatively low concentrations. The method, which involves two derivatization reactions, has been validated previously (42), but the analysis of more than 700 samples in the present trial was a challenge. The significant correlation of [pyridine-D_4]hydroxy acid with total [pyridine-D_4]NNAL (Spearman coefficient = 0.79, $P<0.001$, Table S2), comparing data obtained by different analysts who were blind to the origin of the samples, strongly supports the validity of the analytical chemistry methods used here.

While the metabolic ratio [pyridine-D_4]hydroxy acid: total[pyridine-D_4]NNAL is a good indicator of NNK/NNAL metabolism by α-hydroxylation, it is not the ideal biomarker. It would have been better to quantify the DNA adducts that result from α-hydroxylation of NNK and NNAL. While these have been well characterized and quantified in rats and mice treated with NNK (8,50), robust methods for their quantitation in available human specimens such as oral cells or blood are not available. The primary metabolites resulting from α-hydroxylation, [pyridine-D_4]keto alcohol and [pyridine-D_4]diol, might also have been better biomarkers of this process than [pyridine-D_4]keto acid and [pyridine-D_4]hydroxy acid that are further downstream, but we were unable to detect these metabolites in the urine of our subjects.

Although the reduction in the [pyridine-D_4]hydroxy acid: total [pyridine-D_4]NNAL ratio was modest in this study, we note that the extent of PEITC's inhibition of tumor formation by NNK can be substantially greater than the inhibition of metabolism or adduct formation. In one study in rats, we observed that PEITC completely inhibited lung tumor formation by NNK, but the corresponding inhibition of NNK-hemoglobin adducts, a biomarker of its metabolic activation, was only about 40% (12). Similar observations have been reported in studies of inhibition of aflatoxin carcinogenesis in rats (51). Therefore, the ultimate effect of PEITC on NNK-induced lung cancer could be greater than predicted by a simple metabolic ratio.

In summary, we report promising results of the first clinical trial of PEITC as an inhibitor of carcinogen metabolism in smokers. We observed a modest, specific, and significant modulation of metabolism of the tobacco-specific lung carcinogen NNK in a direction that would predict efficacy in protection against cancer. Our results suggest some potentially important modifications in study design related to dose, dosage form, and length of the dosing period that could lead to greater efficacy in future trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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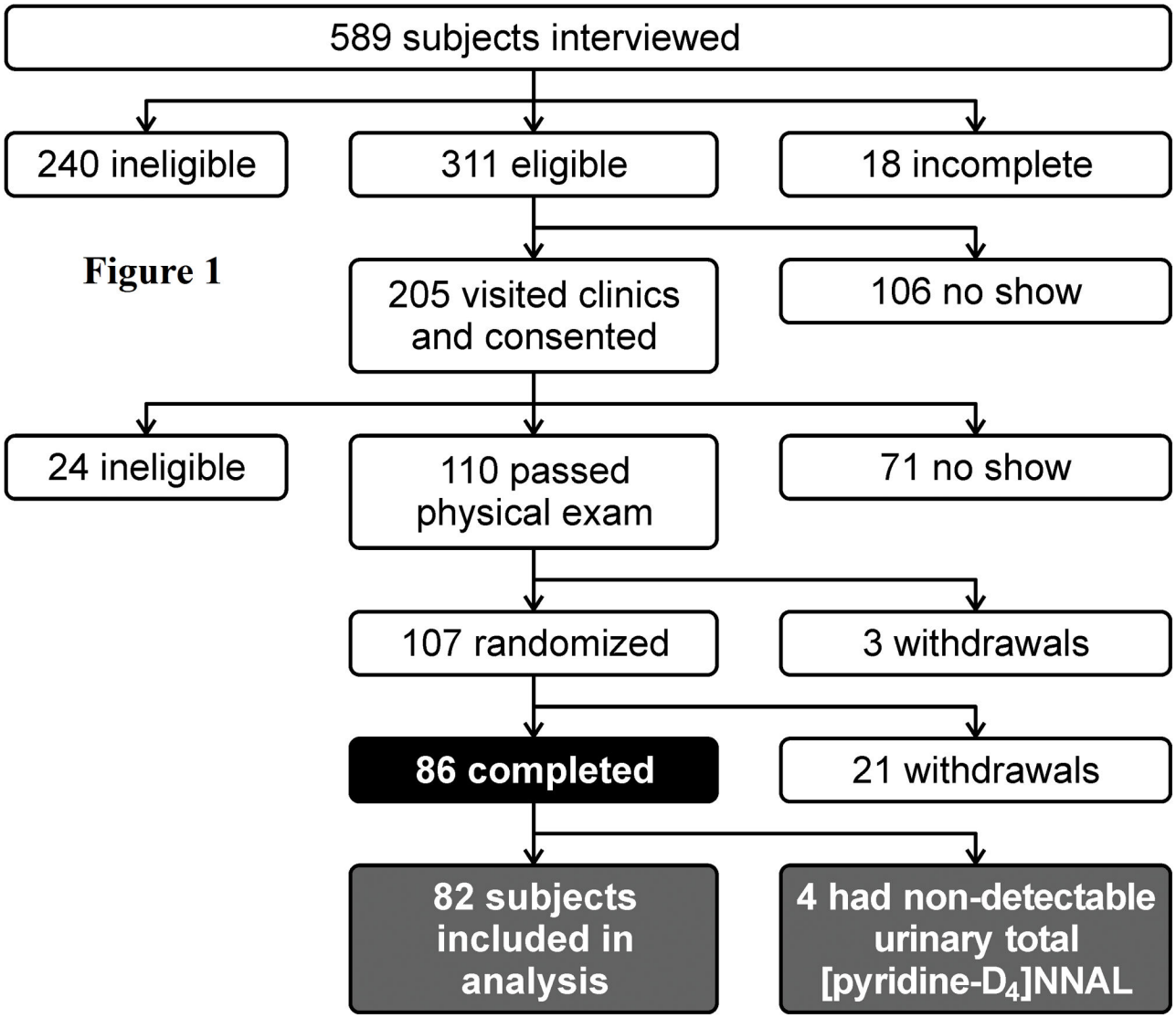

Figure 1.
Profile of subject flow in the PEITC trial.

Table 1

Distributions of demographics and lifestyle factors and *glutathione-S-transferase* (*GST*) genotypes of study subjects by the treatment sequence assignment The PEITC Intervention Study 2008–2013

Characteristics at baseline[a]	Treatment sequence assignment			Total
	PEITC-Placebo	Placebo-PEITC	*P*[b]	
Number of subjects	41	41		82
Age (years), mean (SD)	40.9 (10.6)	41.1 (9.6)	0.939	41.0 (10.1)
Body mass index (kg/m^2), mean (SD)	28.0 (4.8)	28.0 (6.3)	0.957	28.0 (5.6)
Gender, n (%)			0.376	
Male	24 (59)	20 (49)		44 (54)
Female	17 (41)	21 (51)		38 (46)
Race, n (%)			0.839	
Africa American	8 (20)	10 (24)		18 (22)
Caucasian American	28 (68)	27 (66)		55 (67)
Others	5 (12)	4 (10)		9 (11)
Level of education, n (%)			0.179	
High school or lower	14 (34)	20 (49)		34 (41)
College or higher	27 (66)	21 (51)		48 (59)
Cigarette smoking, mean (SD)				
Cigarettes/day	19.3 (6.6)	19.0 (6.7)	0.843	19.1 (6.6)
Years of smoking	14.7 (10.3)	17.9 (9.4)	0.157	16.3 (9.8)
Age at starting smoking (year)	15.2 (4.9)	15.1 (4.6)	0.963	15.1 (4.7)
Age became regular smokers (year)	18.6 (7.1)	17.7 (5.2)	0.514	18.1 (6.2)
Alcohol drinking, n (%)[†]			0.784	
Never	14 (35)	17 (42)		31 (39)
Monthly or less	14 (35)	12 (30)		26 (32)
Greater than monthly	12 (30)	11 (28)		23 (29)
GSTM1 & *GSTT1* genotypes, n (%)			0.585	
Both present	20 (49)	18 (44)		38 (46)
Present/null	3 (7)	4 (10)		7 (9)
Null/present	14 (34)	11 (27)		25 (30)
Both null	4 (10)	8 (19)		12 (15)

[a] Abbreviations: SD, standard deviation.

[b] 2-sided *P* for the difference between the two treatment assignment groups based on the two-group *t*-test (for continuous variables) or χ^2 test (for nominal or categorical variables).

[†] Two subjects were excluded from this analysis due to missing data.

Table 2

Distributions of urinary metabolites of nicotine, NNK and [pyridine-D_4]NNK of study subjects by the treatment sequence assignment at the end of smoking adaptation (visit #2) The PEITC Intervention Study 2008–2013

Urinary metabolites of nicotine and NNK	Treatment sequence assignment			Total
	PEITC-Placebo	Placebo-PEITC	*P*[a]	
Number of subjects	40[b]	41		82
[pyridine-D_4]NNK cigarettes/day, mean (SD)[c]	21.6 (8.5)	20.1 (7.1)	0.419	20.9 (7.8)
Urinary metabolites, geometric mean (95% confidence interval)				
Total nicotine, ng/mg Cr	2114 (1670,2676)	3015 (2389,3806)	0.039	2530 (2136, 2997)
Total cotinine, ng/mg Cr	2839 (2368, 3402)	3371 (2819, 4031)	0.190	3096 (2725, 3519)
Total 3′-hydroxycotinine, ng/mg Cr	6638 (5355, 8227)	6993 (5657, 8646)	0.736	6815 (5866, 7919)
Total nicotine equivalents, nmol/mg Cr	45.1 (37.6, 54.0)	58.1 (48.6, 69.5)	0.054	51.2 (45.0, 58.3)
Total 3′-hydroxycotinne:total cotinine ratio	2.34 (1.96, 2.79)	2.07 (1.74, 2.47)	0.348	2.20 (1.94, 2.49)
Total NNAL, pmol/mg Cr	0.85 (0.65, 1.10)	0.94 (0.73, 1.22)	0.567	0.89 (0.75, 1.07)
Free NNAL, pmol/mg Cr	0.24 (0.19, 0.30)	0.29 (0.23, 0.36)	0.276	0.26 (0.22, 0.31)
Total [pyridine-D_4]NNAL, pmol/mg Cr	0.24 (0.19, 0.31)	0.26 (0.20, 0.33)	0.697	0.25 (0.21, 0.30)
Free [pyridine-D_4]NNAL, pmol/mg Cr	0.08 (0.06, 0.10)	0.09 (0.07, 0.11)	0.546	0.08 (0.07, 0.10)
[Pyridine-D_4]hydroxyl acid, pmol/mg Cr	0.11 (0.09, 0.14)	0.13 (0.11, 0.17)	0.262	0.12 (0.11, 0.14)
[pyridine-D_4]hydroxy acid:total [pyridine-D_4]NNAL ratio	0.47 (0.39, 0.55)	0.52 (0.44, 0.62)	0.398	0.49 (0.44, 0.56)

[a] 2-sided *P* for the difference between the two treatment assignment groups based on the two-group *t*-test.

[b] One subject did not provide the 24-hr urine sample at visit 2, thus, were excluded from the analysis.

[c] Six subjects were excluded from analysis due to missing data (1 in PEITC-Placebo and 5 in Placebo-PEITC group); SD, standard deviation.

Table 3

Urinary levels of [pyridine-D_4]NNK metabolites by PEITC treatment, The PEITC Intervention Study 2008–2013

Urinary metabolites of [pyridine-D_4]NNK	Geometric mean [pmol/mg creatinine]			
	Placebo (n= 82)	PEITC (n=82)	% difference (95% CI)	P for difference[a]
Total [pyridine-D_4]NNAL (pmol/mg creatinine)	0.336	0.336	–0.1 (–8.5, 9.1)	0.980
[pyridine-D_4]Hydroxy acid (pmol/mg creatinine)	0.150	0.140	–6.7 (–13.8, 1.0)	0.092
[pyridine-D_4]Hydroxy acid: [pyridine-D_4] total NNAL ratio	0.455	0.420	–7.7 (–13.8, –1.2)	0.023

[a] 2-sided *P* values were for PEITC treatment effect and derived from the mixed model.

Table 4

Urinary levels of [pyridine-D_4]hydroxy acid:total [pyridine-D_4]NNAL ratio by PEITC treatment stratified by *GSTM1* and *GSTT1* genotypes, The PEITC Intervention Study 2008–2013

GST genotypes	No	Geometric means of [pyridine-D_4]hydroxy acid:total [pyridine-D_4]NNAL ratio				P for interaction[b]
		Placebo	PEITC	% difference (95% CI)	P value[a]	
GSTM1						0.694
null	37	0.423	0.397	−6.2 (−16.5, 5.3)	0.284	
Present	45	0.483	0.441	−8.8 (−15.9, −1.1)	0.031	
GSTT1						0.574
null	19	0.387	0.344	−11.1 (−19.7, −1.6)	0.037	
Present	63	0.478	0.446	−6.7 (−14.2, 1.5)	0.111	
GSTM1 & GSTT1						0.436
Both null	12	0.373	0.314	−15.6 (−26.7, −2.9)	0.039	
Only one gene present	32	0.445	0.431	−3.1 (−14.6, 9.9)	0.623	
Both genes present	38	0.496	0.450	−9.4 (−17.4, −0.6)	0.045	

[a] 2-sided *P* values were derived from the mixed models to test PEITC treatment effect within specific GST genotype group.

[b] 2-sided *P* values were derived from the mixed models to test the modifying effect of genotype on the PEITC's effect on the [pyridine-D4]hydroxy acid:total [pyridine-D4]NNAL ratio.

Table 5

Urinary levels of [pyridine-D_4]hydroxy acid:total [pyridine-D_4]NNAL ratio by PEITC treatment in stratified analysis, The PEITC Intervention Study 2008–2013

Stratifying variable at baseline (visit 2)	No	Geometric means of [pyridine-D_4]hydroxy acid:total [pyridine-D_4]NNAL ratio				P for interaction[b]
		Placebo	PEITC	% difference (95% CI)	P value[a]	
Age						0.245
< 40	34	0.531	0.515	−3.2 (−12.9, 7.7)	0.559	
≥ 40	48	0.408	0.364	−10.8 (−18.3, −2.5)	0.015	
Sex						0.606
Men	44	0.474	0.446	−6.0 (−15.1, 4.0)	0.238	
Women	38	0.434	0.394	−9.3 (−17.2, −0.7)	0.043	
Years of smoking						0.675
≤10	22	0.511	0.465	−9.0 (−21.8, 6.0)	0.240	
11–20	30	0.441	0.423	−4.2 (−13.0, 5.4)	0.386	
≥ 21	30	0.436	0.385	−11.6 (−22.1, 0.2)	0.065	
Total nicotine equivalents[c]						
< median	40	0.507	0.453	−10.7 (−18.8, −1.7)	0.026	0.544
≥ median	41	0.409	0.381	−6.8 (−15.8, 3.3)	0.186	
Total 3′-hydroxycotinine: total cotinine ratio[c]						0.345
< median	40	0.463	0.438	−5.6 (−14.0, 3.8)	0.243	
≥ median	41	0.446	0.395	−11.5 (−19.6, −2.4)	0.018	

[a] 2-sided *P* values were derived from the mixed models to test PEITC treatment effect within a subgroup by stratifying variable.

[b] 2-sided *P* values were derived from the mixed models to test PEITC treatment effect cross group of stratifying variable.

[c] One subject did not provide the 24-hr urine sample at visit 2, thus, were excluded from the analysis.