Establishment and Application of Methods for the Detection of DNA and Protein Adducts from Tobacco-Specific Nitrosamines and Benzo[*a*]pyrene

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This thesis is dedicated

to my future son

Sebastian,

with a father's love.

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Abbreviations

AC, adenocarcinoma

*c.t.*DNA, calf thymus DNA

CV, Coefficient or variance

EH, epoxide hydrolase

ETS, environmental tobacco smoke

HPLC-FD, high performance liquid chromatography-online fluorescence detection

GC-NICI-MS, gas chromatography-negative ion chemical ionization-mass spectrometry

i.p., intraperitoneal

IARC, International Agency for Research on Cancer

ITC, isothiocyanates

LOD, limit of detection

MN, microccal nuclease *S*7

MN/PDE II, mixture of microccal nuclease *S*7 plus phosphodiesterase II (1mg/ml each)

NAC, *N*-acetylcysteine

ND, not detected

NER, nucleotide excision repair

PDE II, phosphodiesterase II

RBC, red blood cells

RT, retention time

SCC, squamous cell carcinoma

s.c., subcutaneous

ZD, zinc-deficient

Chemical names

7-mT, 7-Methylthymidine

B[*a*]P, Benzo[*a*]pyrene

B[*a*]P-7,8-oxide, (7R,8S)-eopxy-7,8-dihydrydroxybenzo[*a*]pyrene.

B[*a*]P-1,8-diol,(7R,8S)-dihydroxy-7,8-dihydrobenzo[*a*]pyrene

B[*a*]P-tetraol,7,8,9,10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (all isomers)

trans/anti B[*a*]P-tetraol, (7R,8S,9S,10R)-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene.

trans/syn B[*a*]P-tetraol, (7R,8S,10R,9R)-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene.

cis/anti B[*a*]P-tetraol, (7S,8R,9S,10R)-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene.

cis/syn B[*a*]P-tetraol, (7S,8R,9R,10S)-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene.

B[*a*]P-TME (7R,8S,9S,10R)-tetramethoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene.

anti-BPDE-(7R,8S)-dihydroxy-(9S,10R)-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene.

BITC, benzyl isothiocyanate

Diol, 4-(3-pyridyl)butane-1,4-diol

HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone (keto alcohol)

Hydroxy acid, 4-hydroxy- acid

Hydroxy aldehyde, 4-(3-pyridyl)-4-oxobutanal

Keto acid, 4-(3-pyridyl)-4-oxobutyric acid

Lactol, 5-(3-pyridyl)-2-hydroxytetrahydrofyran

NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.

NNN, *N*-nitrosonornicotine

NMBA, *N*-nitrosomethylbenzylalamine

O^6-mG, O^6-methylguanine.

PEITC, phenethyl isothiocyanate.

PFBC, pentafluorobenzoyl chloride.

PPITC, 3-phenlypropyl isothiocyanate

Zusammenfassung

Der durch Rauchen hervorgerufene Lungenkrebs ist eine der häufigsten Krebserkrankungen. Während der 70er und 80er Jahr wurden vielfältige Bemühungen die Raucherquoten zu reduziern erfolgreich durchgeführet. Im letzten Jahrzehnt sind die Raucherquoten jedoch unverändert, und es wird vermutet, dass die verbleibenden 25% der Bevölkerung nikotinabhänig sind und auch mit Nikotinersatzmittel (Nikotinkaugummi, Nikotinpflaster) das Rauchen nicht aufgeben können. Eine Alternative um die durch Lungenkrebs hervorgerufenen Sterberaten zu reduzieren, wäre die Anwendung von chemischen Mixturen, die das Krebsrisiko in Risikogruppen (z.B. Raucher und Ex-Rauchern) vermindern. Bevor solche Mixturen allerdings Anwendung finden können, muss die Wirksamkeit, Toxizität und die molekularen Wirkmechanismen der einzelnen aktiven Verbindungen verstanden sein.

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), *N*-Nitrosonornicotine (NNN) und Benzo[*a*]pyrene (B[*a*]P) sind einige der carcinogensten Verbindungen im Zigarettenrauch und werden häufig als Testcarcinogene verwendet. Es wird heutzutage allgemein akzeptiert, dass die kovalente Bindung von Karzinogenen entweder direkt oder nach metabolischer Aktivierung einen entscheidenen Schritt in der Kanzinogenese darstellt. In Tierversuchen hat sich gezeigt, dass Isothiocyanate (ITC) effektiv die Krebsentstehung von NNK, NNN und/oder B[*a*]P hemmen können.

In der vorliegenden Arbeit wurde daher der Einfluss von verschiedenen ITCs auf die Adduktbildung von NNK, NNN und B[*a*]P untersucht. Zu Beginn wurden HPLC-FD und GC-NICI-MS Methoden zur Bestimmung von DNA- und Proteinaddukten von NNK, NNN und B[*a*]P im Labor etabliert. Diese Methoden wurden dann in verschiedenen Tierstudien angewendet.

Erstens wurde gezeigt, dass die Adduktbildungen von NNK und B[*a*]P sich nicht gegenseitig beeinflussen. Die Werte von HPB-freilassenden, O^6-mG und B[*a*]P-Tetraol-freilassenden Addukten waren gleich, unabhängig ob NNK und B[*a*]P zusammen oder einzelnd verabreicht wurden. Die selben Behandlungen wurden dann verwendet, um den

Einfluss von verschidenen Mixturen von PEITC und BITC auf die Adduktbildung in mit NNK und B[*a*]P behandelten Mäusen und Ratten zu untersuchen.

PEITC reduziertet die HPB-freilassenden-DNA Adduktwerte um 40-50%. Dies war signifikant, wenn PEITC im Futter gemischt verabreicht wurde, jedoch nicht, wenn ITCs einmal in der Woche per orale Intubation verabreicht wurden. Vorher wurde gezeigt, dass die verwendetet ITC Behandlungen die Lungentumore um ebenfalls 40% reduziert. Die O^6-mG-Werte waren nur gering beeinflusst durch die untersuchten ITC-Behandlung. B[*a*]P-Tetraol-freilassenden Adduktwerte waren 120 Stunden nach der letzten Behandlung mit NNK plus B[*a*]P reduziert. In der Rattenstudie wurde NNK im Trinkwasser und B[*a*]P, PEITC und BITC im Futter verabreicht. PEICT reduzierte die HPB-freilassenden DNA Addukte um 50% in der Lunge und war wirkungslos in der Leber. Intressanterweise wurde eine Reduktion der HPB-freilassenden Adduktwerte in den Mäusebehandlungen nur dann beobachtet, wenn die Kontrollen einen Adduktwert von grösser als 1 fmol/µg DNA hatten. In den Ratten waren die reduzierten Adduktwerte in der Lunge ähnlich zu den Werten in der Leber. Diese Beobachtungen lassen vermuten, dass in Mäusen und Ratten mindestens zwei Aktivierungssysteme für NNK vorhanden sind. Das eine ist PEITC-sensitiv und vermutlich verantwortlich für die grösseren Adduktwerte und Tumorzahlen in der Lunge. Das andere ist PEITC-unsensitiv und verursacht vermutlich die nach PEITC Behandlung verbleibenden Adduktwerte und Tumore hervor.

In der letzten Tierstudie wurden der Einfluss von PEITC, PPITC und ihren NAC-Konjugaten auf HPB-freilassende DNA- und Globinaddukte von NNN in der Ratte untersucht. Keine der untersuchten Behandlungen hatte einen Einfluss auf die Bildung von Addukten, auch wenn vorher gezeigt wurde, dass die verwendeten Behandlungen eine starke Reduzierung von Speiseröhrentumoren bewirkt. Die Ergebnisse haben gezeigt, dass die Verminderung von DNA Addukten nur teilweise den krebsvorbeugende Mechanismus von ITCs darstellt und andere Mechanismen ebenfalls beteiligt sind. Neuere Erkenntnisse lassen vermuten, dass die Aktivierung von Apoptosis — programmierter Zelltod — möglicherweise für die krebsvorbeugende Wirkung von ITCs verantwortich ist. Dies muss jedoch durch zusätzliche Studien bewiesen werden.

Summary

Lung cancer, mainly caused by tobacco smoke, is the leading cause of cancer mortality. Large efforts in prevention and cessation have reduced smoking rates in the U.S. and other countries. Nevertheless, since 1990, rates have remained constant and it is believed that most of those currently smoking (~25%) are addicted to nicotine, and therefore are unable to stop smoking. An alternative strategy to reduce lung cancer mortality is the development of chemopreventive mixtures used to reduce cancer risk. Before entering clinical trails, it is crucial to know the efficacy, toxicity and the molecular mechanism by which the active compounds prevent carcinogenesis.

4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), *N*-nitrosonornicotine (NNN) and benzo[*a*]pyrene (B[*a*]P) are among the most carcinogenic compounds in tobacco smoke. All have been widely used as model carcinogens and their tumorigenic activities are well established. It is believed that formation of DNA adducts is a crucial step in carcinogenesis. NNK and NNN form 4-hydroxy-1-(3-pyridyl)-1-butanone releasing and methylating adducts, while B[*a*]P forms B[*a*]P-tetraol-releasing adducts. Different isothiocyanates (ITCs) are able to prevent NNK-, NNN- or B[*a*]P-induced tumor formation, but relative little is know about the mechanism of these preventive effects. In this thesis, the influence of different ITCs on adduct formation from NNK plus B[*a*]P and NNN were evaluated.

Using an A/J mouse lung tumor model, it was first shown that the formation of HPB-releasing, O^6-mG and B[*a*]P-tetraol-releasing adducts were not affected when NNK and B[*a*]P were given individually or in combination, of by gavage. Using the same model, the effects of different mixtures of PEITC and BITC, given by gavage or in the diet, on DNA adduct formation were evaluated. Dietary treatment with phenethyl isothiocyanate (PEITC) or PEITC plus benzyl isothiocyanate (BITC) reduced levels of HPB-releasing adducts by 40–50%. This is consistent with a previously shown 40% inhibition of tumor multiplicity for the same treatment. In the gavage treatments with ITCs it seemed that PEITC reduced HPB-releasing DNA adducts, while levels of BITC counteracted these effects. Levels of O^6-mG were minimally affected by any of the treatments. Levels of B[*a*]P-tetraol releasing adducts were reduced by gavaged PEITC and BITC, 120 h after the last carcinogen treatment, while dietary treatment had no effects. We then extended our investigation to F-344 rats by using a similar ITC treatment protocol as in the mouse model. NNK was given in the drinking water and B[*a*]P in diet. Dietary PEITC reduced the formation of HPB-releasing globin and DNA adducts in lung but not in liver, while levels of B[*a*]P-tetraol-releasing adducts were unaffected. Additionally, the effects of PEITC, 3-phenlypropyl isothiocyanate, and their *N*-acetylcystein conjugates in diet on adducts from NNN in drinking water were evaluated in rat esophageal DNA and globin. Using a protocol known to inhibit NNN-induced esophageal tumorigenesis, the levels of HPB-releasing adduct levels were unaffected by the ITCs treatment.

The observations that dietary PEITC inhibited the formation of HPB-releasing DNA adducts only in mice where the control levels were above 1 fmol/µg DNA and adduct levels in rat lung were reduced to levels seen in liver, lead to the conclusion that in mice and rats, there are at least two activation pathway of NNK. One is PEITC-sensitive and responsible for the high adduct levels in lung and presumably also for higher carcinogenicity of NNK in lung. The other is PEITC-insensitive and responsible for the remaining adduct levels and tumorigenicity.

In conclusion, our results demonstrated that the preventive mechanism by which ITCs inhibit carcinogenesis is only in part due to inhibition of DNA adduct formation and that other mechanisms are involved. There is a large body of evidence indicating that induction of apoptosis may be a mechanism by which ITCs prevent tumor formation, but further studies are required.

1 Introduction

Cancer, a long known deadly disease, is still the most challenging disease in medical research. It is characterized by the uncontrolled growth and spread of abnormal cells. Scientists widely accept that multiple changes in DNA modifying gene expression are responsible for tumor development (1,2). These genetic modifications are caused by biological, physiological or chemical factors of endogenous or exogenous origin. As early as 1875, observation of high incidences of skin cancer in chimney sweeps (3) and tar distillery workers (4) suggested that occupational exposure may be responsible for tumor development. In the 1930's Kenneway identified B[*a*]P as the major carcinogenic compound in coal tar (5) and set the stage for chemical carcinogenesis. Since then, epidemiological studies, in combination with modern analytic techniques on environmental and occupational exposures, have identified a variety of chemical carcinogens. Prominent examples include the link between tobacco smoke and lung cancer (6,7), 4-aminobiphenyl and bladder cancer (8) and aflatoxin and liver cancer (9). Studies in animal models revealed that many chemical carcinogens require metabolic activation to exhibit their carcinogenic potential (Figure 1). Chemically the first step in cancer initiation is the covalent binding of a carcinogen or its activated metabolites to DNA, forming adducts (10,11). In *in vitro* and *in vivo* experiments, DNA adducts cause mutations during DNA replication or mismatch repair when not repaired in time (12-14). Most mutations are silent, but when they cause a change in the amino acid sequence in an essential gene, the cell may undergo apoptosis — programmed cell death. Mutations that occur in a critical region of a cancer related gene however can activate oncogenes or deactivate the tumor suppressor genes. Multiple events of this type lead to aberrant loss of cell control and ultimately to cancer. Studies on tumor tissues revealed clearly that the formation of mutations is a critical step in tumor development. High mutation frequencies in specific genes were identified (15,16). Based on these observations, it is widely accepted that chemical carcinogens initiate and promote cancer through adduct formation leading to mutation (Figure 1). The accumulation of mutations can lead to aberrant cells with loss of normal growth control and may cause cancer (12).

During the last two decades, methods were developed to measure carcinogen metabolites and carcinogen DNA and protein adducts in animals and humans. The analysis of carcinogen adducts or metabolites in urine, blood or tissues represent biological endpoints. Markers for these endpoints — biomarkers — are useful tools in the analysis of metabolic activation and detoxification of carcinogens. The analysis of DNA adducts *in vivo* represents a biological endpoint that accounts for absorption, metabolic activation and detoxification, and DNA repair. DNA adduct measurement provides important information on mechanisms of carcinogenesis. Animal studies give insights on the relationship of carcinogen exposure, DNA adduct formation and carcinogenesis. This knowledge can potentially be used to identify populations or subjects that are at higher risk for cancer induced by a specific carcinogen. Furthermore, differences in race, polymorphism, gender or lifestyles that may increase cancer susceptibility can be identified and preventive measures can be applied.

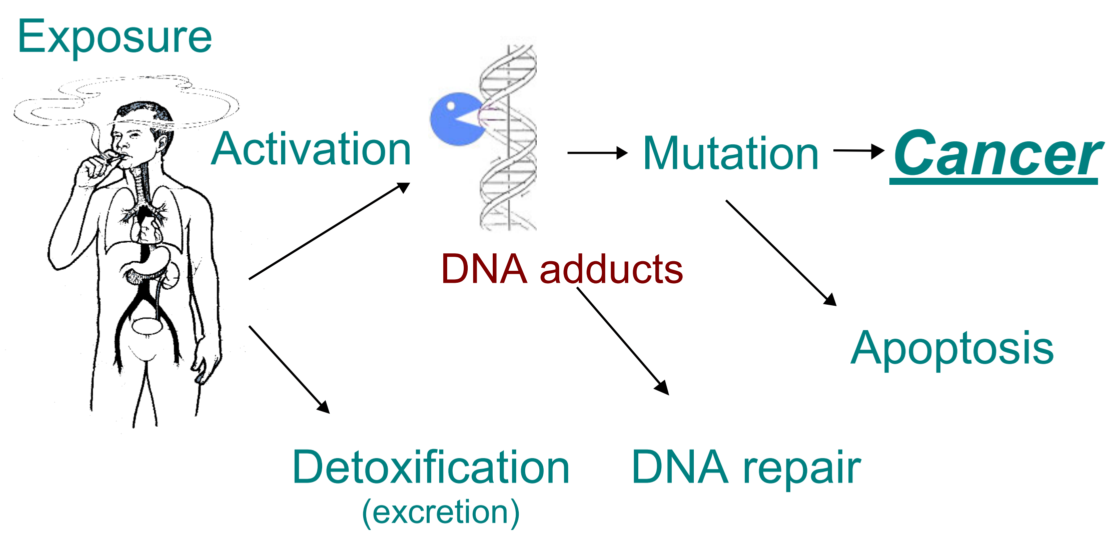


Figure 1: Mechanistic scheme of cancer initiation.

1.1 Lung and Esophageal cancer

Lung cancer is the leading cause of avoidable cancer death. The five year survival rate of lung cancer is 15%. This is much lower than that of other major cancers such as colon (61%), breast (86%) or prostate (96%) (17). It is estimated that in the United States, more than 155,000 people will die of lung cancer in 2002 (17). Epidemiological studies clearly identify tobacco usage as the main cause for lung cancer and cancers in the upper aero-digestive tract. Compared to lifelong never-smokers, the lung cancer mortality rate is about 22 times higher for male and 12 times higher for female smokers (18). The risk of lung cancer reduces after smoking cessation, however not during the first 5 years and never to that of a lifetime never smoker (19). In addition, exposure to environmental tobacco smoke is a widely accepted cause of lung cancer, although the risk is much lower and it is difficult to assess (20,21). The relative risk of lung cancer for non-smokers living with a smoking spouse or family member is 20% higher than that of those not exposed to ETS at home (20).

Esophageal cancer has received considerable attention because over the past 20 years, the number of new cases has tripled (22). In contrast to lung cancer, where tobacco usage has been clearly identified as the main cause, the etiology of esophageal carcinogenesis is as not as clear. Risk factors for esophageal cancer are tobacco, alcohol, salt-pickled, salt-cured and moldy foods, *N*-nitrosamines carcinogens (from multiple sources), vitamins (A, C, E, etc.) and trace mineral (zinc, selenium) deficiencies, hot beverages, fungal invasion of esophageal tissues, human papiloma virus infections or heritable susceptibility genes (reviewed in (23,24)). In U.S. and Europe, the strongest etiology factors appear to be smoking and alcohol consumption. Since the five-year survival rate is only ~14%, understanding the mechanism is in crucial (25). In a widely used rat esophagus tumor model, several asymmetric nitrosamines were found to be strong inducers of esophageal tumors (26).

1.2 Chemical carcinogens in tobacco smoke

Cigarette smoke is composed of a vapor phase and a particle phase. The vapor phase accounts for about 95% of smoke is made up mainly of nitrogen, oxygene, and carbon dioxide (27). Over sixty carcinogens in cigarette smoke have shown "sufficient evidence for carcinogenicity" in animals or humans as determined by the International Agency for Research on Cancer (IARC) (summarized in (28)). Other carcinogens not evaluated by IACR with respect to their carcinogenicity may also be present. Table 1 shows a selected overview of compound classes in cigarette smoke and approximate levels of some single compounds classified as carcinogens. *N*-Nitrosamines and polycyclic aromatic hydrocarbons (PAH) are among the classes of compounds that show the strongest carcinogenic potential in tobacco smoke (29). The ratio of adenocarcinoma (AC) to squamous cell carcinomas (SSC) of the lung in smokers has been increasing over the past three decades correspondingly to the change in the composition o cigarettes (30). The increase in NNK, which induces AC, and the decrease in B[*a*]P, which induces SCC, may account in part for the increased ratio.

Table 1: Overview of selected carcinogens in cigarette smoke

Compound class/compound	**In mainstream smoke [ng/cigarette]**
Polycyclic aromatic hydrocarbons (10)[a]	
Benzo[*a*]pyrene (B[*a*]P)	20-40
Heterocyclic amines (6)	
N-Nitrosamines (10)	
N-Nitrosonornicotine (NNN)	120-3700
4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)	80-770
Aromatic amines (13)	
Aldehydes (2)	
Phenolic compounds (3)	
Volatile hydrocarbons (3)	
Miscellaneous organic compounds (10)	
Inorganic compounds (9)	

[a] Number in bracket indicates number of compounds classified as carcinogenic by IARC (28).

1.3 Tobacco-specific *N*-nitrosamines

Extensive studies demonstrate that tobacco-alkaloid-derived nitrosamines, called tobacco-specific *N*-nitrosamines, are present in substantial quantities in tobacco, tobacco smoke, and smokeless tobacco (reviewed in (28)). Among these, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is a potent lung carcinogen in all models studied. NNK induces mainly adenomas and adenocarcinomas in lung, independent of the route of administration (31). NNK is rapidly reduced to NNAL and its presents in human urine, free or as glucuronide-conjugate, has been used as valuable biomarker for exposure to NNK as representative of tobacco smoke (32-34). This metabolite has been determine in variety of studies demonstrating that not only smoker are exposed to NNK, but individuals exposed ETS at the work place and at home (33,35-37). While it is unlikely that a single carcinogen or group of carcinogen are responsible for a specific cancer data suggest that NNK and NNN are responsible of the lung, oral cavity, esophagus and pancreas (38).

1.3.1 Metabolic activation of NNK

NNK can be metabolized via the following five pathways: carbonyl reduction, pyridine oxidation, α-hydroxylation (hydroxylation of the carbons adjacent to the *N*-nitroso group), denitrosation and formation of ADP adducts. The major pathway of NNK activation to DNA adducts is via α-hydroxylation. α-Hydroxylation of NNK at the methyl carbon produces α-hydroxymethyl-NNK, which is stable enough to undergo glucuronidation (39). The formation of this glucuronide could either serve as a detoxification pathway or provide a stable transport form of the α-hydroxylated metabolite. α-Hydroxmethyl-NNK spontaneously loses formaldehyde producing a pyridyloxobutyldiazohydroxide, which reacts with DNA and proteins (Figure 2, (40)). O^6-Pyridyloxobutyl-deoxyguanosine (O^6-POB-dG), one of the DNA adducts formed in this reaction, is highly mutagenic in Escherichia coli strain DH10B and human kidney cells (41). Other adducts formed by this pathway are still under investigation and different structures have been proposed (40). *In vivo* and *in vitro* studies have shown that the major DNA adduct formed by this pathway, accounting for about 50% of the bound material, releases HPB upon acid or neutral hydrolysis, but not under base hydrolysis. (42-47).

α-Hydroxylation at the methylene carbon yields α-methylenehydroxy-NNK, which spontaneously produces methanediazohydroxide and a keto aldehyde. Methanediazohydroxide reacts with DNA to form 7-methylguanine (7-mG), O^6-methylguanine (O^6-mG), and O^4-methylthymidine adducts. Animals studies have clearly revealed a correlation between methylating or pyridyloxobutylating adducts and lung tumorigenesis by NNK (42,48,49).

Since the first detection of O^6-mG and 7-mG in the liver and lung of NNK treated F-344 rats (50), there has been substantial research on the occurrence and biological significance of methyl and pyridyloxobutyl (HPB-releasing) adducts derived from NNK. Adduct measurements provide important information on the mechanism of NNK-induced carcinogenesis. With a few exceptions, adduct formation occurs mainly in target tissues of NNK carcinogenesis: lung, nasal mucosa, and liver (31).

In mice, lung tumors are induced rapidly by a single dose of 10 µmol NNK (51). This model was used extensively to examine molecular mechanism as well as the effects of chemopreventive agents. Levels of 7-mG are greater than O^6-mG and both are higher than HPB-releasing adducts (42). Methylating adduct levels peak 4 h after injection of 10 µmol NNK, while HPB-releasing adduct levels are highest at 24 h (43). It is proposed that multiple cytochrome P450s are involved in the α-hydroxylation of NNK, causing different kinetics of DNA methylation and pyridyloxobutylation (31). The importance of methylating adducts in mouse lung tumorigenesis was shown by Peterson et al. (43). The levels of O^6-mG correlated strongly with tumor multiplicity, independent of the source of methylating agents. Additionally, GC-TA transition mutations in K-*ras* oncogene observed in NNK-induced lung tumors are consistent with the importance of O^6-mG (52).

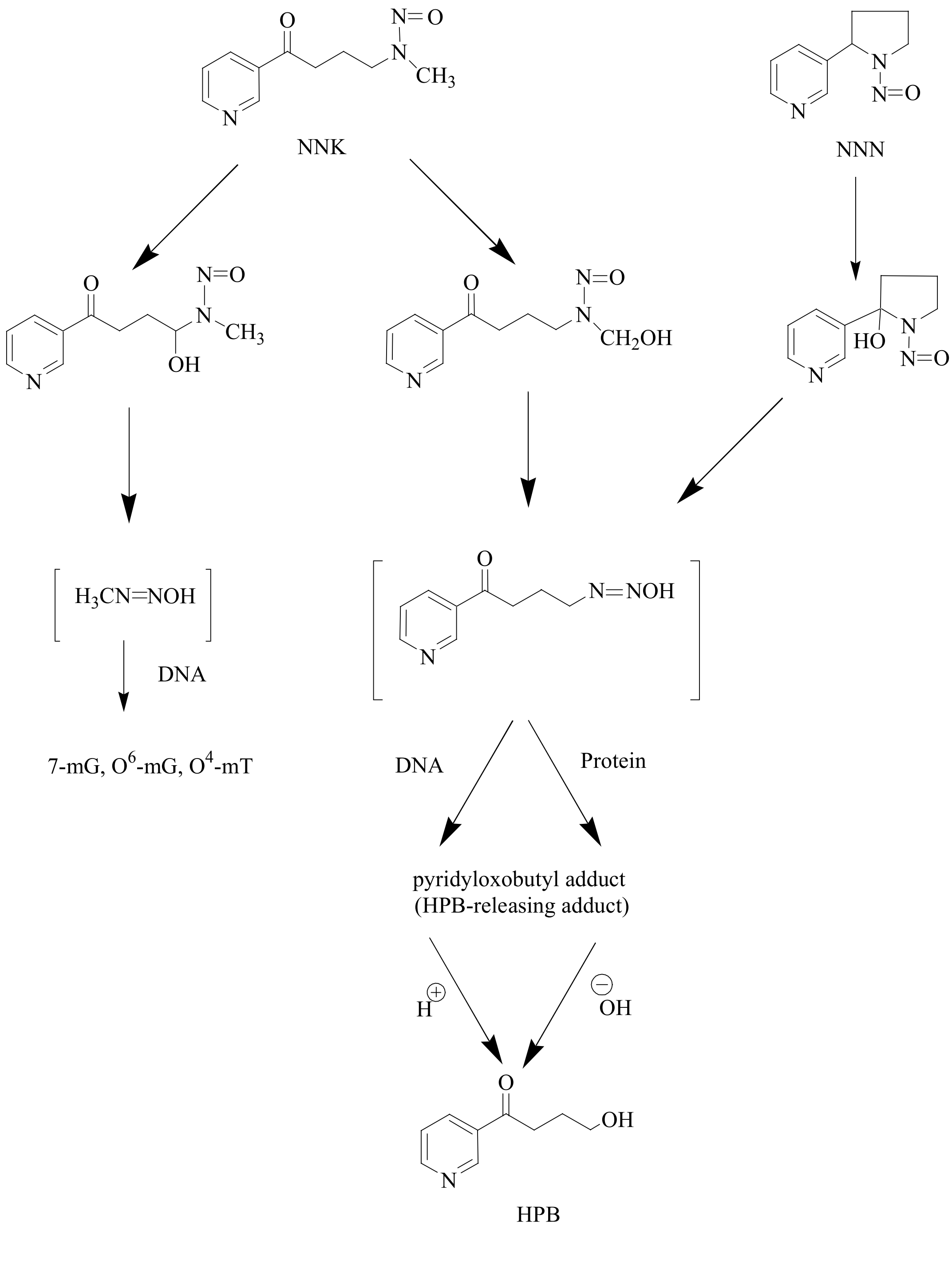


Figure 2: Metabolic activation of NNK and NNN and the formation of DNA and globin adducts.

1.3.2 Metabolic activation of NNN

The metabolism of NNN follows three types of reactions: pyridine N-oxidation producing NNN-*N*-oxide, α-hydroxylation, and formation of norcotinine. NNN-*N*-oxide is a detoxification product. NNN-*N*-oxide is observed in rat liver at substantial amounts, while in extahepatic tissues, its formation is limited or non-existent (53-56). In rats, NNN-*N*-oxide formation accounts for 7-11% of NNN metabolites excreted in urine (57,58). In human liver microsome preparations, NNN-*N*-oxide formation has not been observed (59,60).

Similar to NNK, the major activation pathway of NNN is through α-hydroxylation. α-Hydroxylation of NNN at the 2'-carbon produces 2'-hydroxy-NNN that spontaneously loses HONO yielding myosmine, or ring-opens to produce 4-(3-pyridyl)-4-oxobutane 1-diazohydroxide (Figure 2). This is the same intermediate formed upon α-methyl hydroxylation of NNK (61,62). The metabolites ultimately formed by this pathway are HPB (keto alcohol), 4-(3-pyridyl)butane-1,4-diol (diol), and 4-(3-pyridyl)-4-oxobutyric acid (keto acid). The sum of these metabolites has been used to quantify the extent of 2'-hydroxylation (55,56,63-66). This common pathway in NNK and NNN metabolism apparently leads to the same adducts with DNA and globin as formed from NNK (44,64,67,68). Adduct formation from NNN has only been observed by this pathway.

α-Hydroxylation at the 5'-carbon leads to 5'-hydroxy-NNN, which spontaneously ring-opens to form 1-(3-pyridyl)-4-oxobutane 1-diazohydroxide (62,69). This reacts with H_2O producing 4-(3-pyridyl)-4-oxobutanal (hydroxy aldehyde) that cyclizes to 5-(3-pyridyl)-2-hydroxytetrahydrofyran (lactol). Adducts from this pathway have not been identified. Whether 5'-hydroxylation is an activation or detoxification pathway is not clear.

In rat liver microsomes, 2'- and 5'-hydroxylation are dependent on NADPH, inhibited by CO, and is inducible or inhibitable by pretreatment with 3-methylcholanthrene, phenobarbital, and Arcolor (62,70,71). P450s appear to be the major catalysts of NNN α-hydroxylation in rat esophagus and nasal mucosa (63,71). The ratio of 2' versus 5'-hydroxylation is typically 2-4 in esophagus, the target tissue of NNN carcinogenesis, while in liver, a non-target tissue, the ratio is 0.2-1.4 (55,66,71). Hydroxy acid and keto acid are the major urinary metabolites of α-hydroxylation of NNN in rats accounting for 40% and 25% of the total dose, respectively (58,72).

β-hydroxylation (hydroxylation at the 4' or 5' carbon) has been reported as a minor pathway in rat hepatic microsomes (54).

In vivo, NNN causes HPB-releasing DNA adducts in rat lung, liver (44,45), and nasal mucosa (67). In binding studies other adducts have been observed in several rat tissues, but were not identified (44,45,73). In contrast to NNK, NNN does not form methylating adducts (e.g., O^6-mG) (74).

NNN binds in vivo to nasal mucosa proteins to a greater extent than that of liver protein (75). This is consistent with the higher activity of rat nasal mucosa for metabolic activation of NNN (63). HPB-releasing adducts, presumably products of 2'-hydroxylation of NNN, are among the hemoglobin adducts formed in NNN treated rats (68). The formation of HPB-releasing globin adducts upon NNN treatment is about 16% that of NNK. HPB-releasing DNA and hemoglobin adducts are good markers for exposure and presumably for metabolic activation via 2'-hydroxylation of NNN in animal models. HPB-releasing adducts have been found in hemoglobin of smokers and snuff dippers, and may be formed by NNK, NNN, or both (76).

When given to F-344 rats, NNN causes tumors mainly in esophagus and nasal cavity (66,77,78). Esophagus is the main target tissue when NNN is given in drinking water, while when injected or given by gavage, nasal mucosa is targeted (77,78). Only limited dose-response data are available. Stoner et al. (72) found a 71% tumor incidence in esophagus with 5 ppm NNN in drinking water, a dose that is much lower than the lowest dose of 0.8-1 mmol/kg tested previously (66,79). This suggests that NNN is a much stronger esophageal carcinogen as predicted from earlier studies with higher doses of NNN.

1.4 B[*a*]P, a polycyclic aromatic hydrocarbon

1.4.1 Metabolic activation of B[*a*]P

PAH are ubiquitous environmental pollutants derived from incomplete combustion of organic materials and are present in tobacco smoke. B[*a*]P is by far the most extensively studied PAH. The period between 1950-1990 witnessed a profusion of research on the mechanism by which B[*a*]P is metabolically activated (80-86). Numerous theories were proposed and ultimately discarded, but one has withstood the tests of time and experimentation; this is outlined in Figure 1. B[*a*]P is metabolically activated via a three-step process (Figure 3). First, cytochromes P450 catalyze the formation of (7*R,8S*)-epoxy-7,8-dihydrobenzo[*a*]pyrene (BaP-7,8-oxide). This is converted to (7*R,*8*R*)-dihydroxy-7,8-dihydrobenzo[*a*]pyrene (BaP-7,8-diol), and catalyzed by epoxide hydrolase. B[*a*]P-7,8-diol then undergoes another oxidation step, catalyzed by cytochromes P450 and other enzymes, producing mainly (7*R,*8*S*)-dihydroxy-(9*S,*10*R*)-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE). Among the four possible 7,8-diol-9,10-epoxide isomers of B[*a*]P, BPDE is formed to the greatest extent in mammalian systems examined to date, and has high tumorigenic activity in murine models (87). BPDE reacts with DNA producing a major adduct at the N^2-position of deoxyguanosine (BPDE-N^2-dG). Convincing evidence clearly documents the presence of this adduct in target tissues of animals treated with B[*a*]P (88). The mutagenicity of BPDE-N^2-dG and its effects on DNA conformation have also been conclusively demonstrated (13,14,89). While other pathways of metabolic activation of B[*a*]P have been proposed, evidence for the production of the relevant DNA adducts *in vivo* is less persuasive (90).

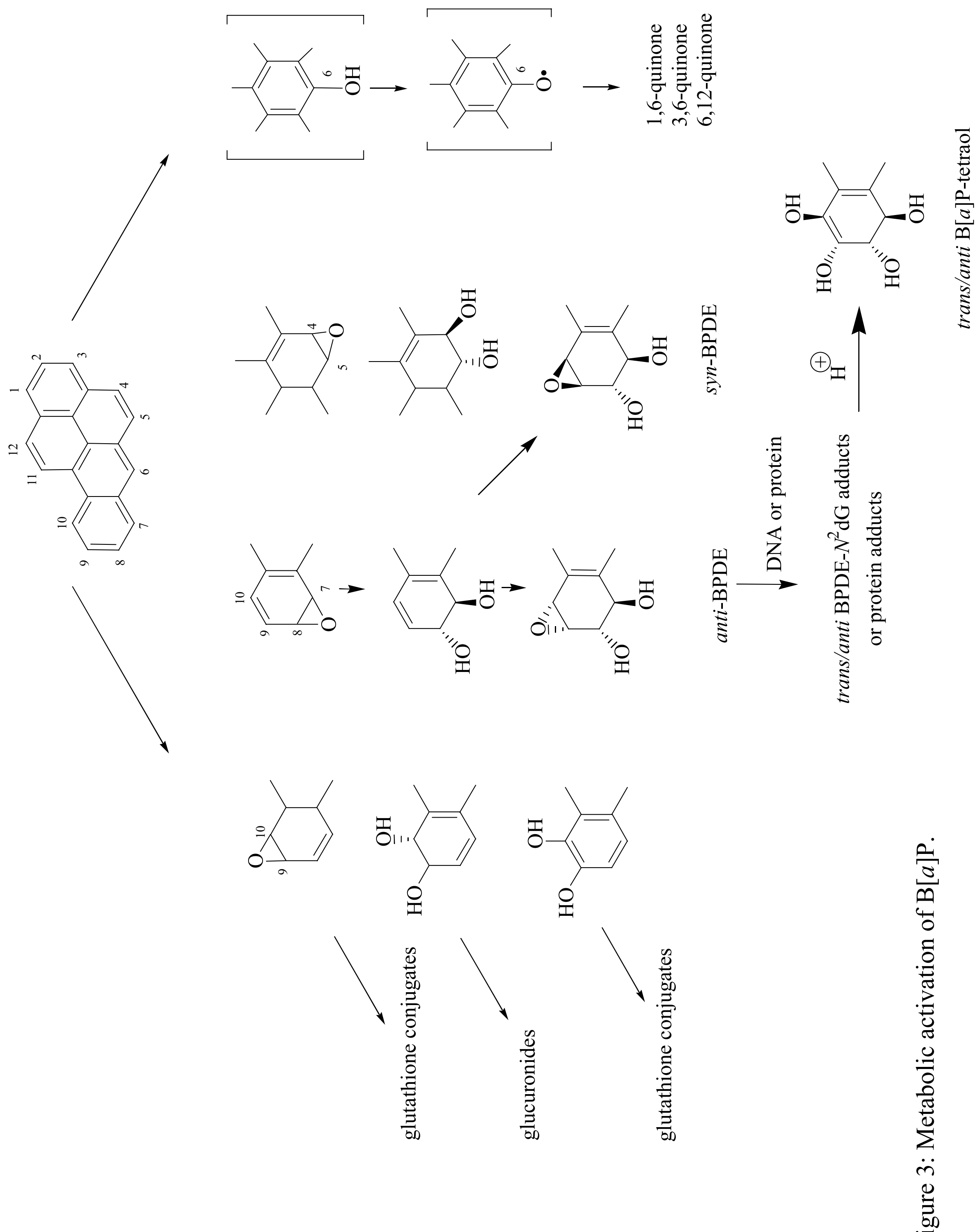


Figure 3: Metabolic activation of B[a]P.

1.4.2 Methodology of BDPE adduct measurement

During the past 20 years, various techniques have been used to measure PAH-DNA adducts in human tissues. The most common among these are ^{32}P-postlabeling and immunoassays (91,92) and reviewed in reference (93) Studies using these techniques have increased our knowledge of carcinogen-DNA adduct formation in different settings where PAH exposure occurs. An advantage of ^{32}P-postlabeling is its sensitivity, with limits of detection (LOD) as low as 1 adduct/ 10^{10} nucleotides when analyzing 12 μg DNA (94). Immunoassays are notable for their relative operational simplicity and are amenable to analysis of multiple samples. However, neither ^{32}P-postlabeling nor immunochemical methods are generally selective enough to identify specific BPDE-DNA adducts. It is well established that antibodies against BPDE-DNA adducts cross react with other PAH-DNA adducts. Thus, these studies cannot provide information specifically on BPDE-DNA adducts (95). ^{32}P-Postlabelling typically detects a mixture of adducts most commonly recognized as a "diagonal radioactive zone" on thin layer chromatography (TLC) plates. These are often referred to as "aromatic DNA adducts" or "hydrophobic DNA adducts", and most likely include BPDE-DNA adducts. Several studies have attempted to quantify one of the TLC spots as BPDE-N^2-dG, but the results are not convincing, as this spot could well contain other material (96,97). In one study, a correlation has been noted between the levels of this adduct as measured by ^{32}P-postlabeling and the more specific high performance liquid chromatography-fluorescence detection (HPLC-FD) method, which may support its identity (98). Generally, ^{32}P-postlabeling with either TLC or HPLC as separation systems, does not provide either qualitative or quantitative data on BPDE-N^2-dG in human tissue DNA. Studies of PAH-DNA adducts using ^{32}P-postlabeling and immunoassays have been reviewed (93,99).

Acid hydrolysis of BPDE-N^2-dG or BPDE-protein adducts produces B[*a*]P-tetraols as illustrated in Figure 3. The isomer shown is the major one detected in studies to date. Most measure BPDE-DNA or protein adducts as released B[*a*]P-tetraols. Early attempts were made to measure B[*a*]P-tetraols released from human DNA using synchronous fluorescence spectroscopy (SFS), but there are interferences by other PAH-DNA adducts (100,101). However, when SFS is combined with immunoaffinity chromatography (IAC) and HPLC, the specificity of the method is improved, although quantitation is still uncertain (96,102-107). The most robust and quantitative methods for measurement of released B[*a*]P-tetraols are HPLC-FD, HPLC-laser fluorescence detection (HPLC-LFD), and gas chromatography-mass spectrometry (GC-MS). The released BP-tetraols are detected directly with an online fluorescence detector (108), or are converted to either methoxyl (B[*a*]P-TME) or trimethylsilyl (B[*a*]P-TMS) derivatives prior to analysis by GC-MS (109,110). The GC-MS techniques generally use deuterated internal standards for quantitation. Due to the detection of the molecular ion, these techniques are more selective than the fluorescence methods, but they are also more time-consuming.

1.5 Prevention

The magnitude of the cancer problem and the nature of conventional therapies such as surgery, radiation and chemotherapy, produced new approaches to control cancer incidence. First, reduction of exposure in occupational and environmental settings has been implicated over the last decades and is expected to reduce cancer incidence in these settings. Studies on occupationally exposed subjects have shown that the reduction of B[*a*]P in ambient air by 40% reduces DNA adduct levels in WBC (111,112). Recently, pharmacological approaches using drugs or micronutrients to reduce cancer risk, and to reverse the process of carcinogenesis, have become popular (reviewed in (113-115)). In the last two of decades may chemopreventive agents have been identified that inhibit carcinogenesis in animal models. Three recent publications demonstrated that this is a serious and practical approach. Tamoxifen (116,117) and raloxofene (118) were shown to be effective in preventing breast cancer in woman at various degrees of risk. The importance of these studies for the future of chemoprevention was that they used a rational approach, by using agents where the molecular effects were well understood. Another rational approach showed that retinol is effective in preventing skin squamous cell carcinoma (SCC), however no effects were observed on advanced SCCs (119)nor did it prevent basal cell carcinoma of the skin. (120).

In contrast, several lung prevention trials with β-carotene/vitamin A (121) unfortunately had no preventive effects and cancer incidences were even increased by β-carotene and vitamin A treatments (122). This failure is perhaps a good example of what can result when putative chemopreventive agents are entered into clinical trails before adequate mechanistic evidence and animal data are available. In regard to lung cancer, vitamin B_{12} plus folic acid (123), fenretinide (124), isotretinoin (119), and etretinate (125) had no effect on cancer incidence. Thus, it is essential to realize that epidemiological data alone do not provide a sufficient basis for the selection of new chemopreventive agents. Therefore, mechanistic studies involving potential chemopreventive agents are in strong demand.

1.5.1 Lung cancer prevention

As mentioned above, smoking accounts for about 87% of all lung cancer cases (17). While efforts in smoking cessation and prevention have reduced smoking rates in the U.S. and other countries beginning in the mid-20th century, smoking prevalence has not changed since 1990 (17). Approximately 25% of the adult population in the U.S. currently smokes (126). It is believed that this persistence of smoking is due to nicotine addiction (29). Cessation may be impossible for the nicotine-addicted smokers, as is shown by the low rates (30% after one year) of successful quitting, even when a nicotine-patch and counseling is applied (127).

An alternative approach to reduce cancer mortality in smokers and ex-smokers may be the development of chemopreventive mixtures that inhibit tobacco-induced lung carcinogenesis. The development of such mixtures is based on epidemiological studies that consistently demonstrate that high vegetable intake reduces the risk for cancers of the lung, mouth, pharynx, stomach, and esophagus (128). The consumption of *Brassica* vegetables has been shown to be preventive against lung cancer in two out of two cohort studies and nine out of nine case-control studies (129). These observations lead to the hypothesis that there are chemopreventive agents present in vegetables. Intensive work identified isothiocyanates (ITC) as a class of compounds that remarkably reduce lung carcinogenesis in animal models (Table 2, (130)). ITC occur naturally in vegetables of the family *Cruciferae*, such as broccoli, brussel sprouts, cauliflower, watercress, horseradish, turnip, and mustard (131). Recently, three epideminologic studies demonstrated protective effects of ITCs against lung cancer (132-134).

1.5.2 The origin of ITC

ITC occur in plants as glucosinolates (thioglucoside conjugates). Thus far, over one hundred glucosinolates have been identified which occur predominantly in vegetables of the family *Cruciferae* (131). Vegetables of the genus *Brassica* represent the main source of glucosinolates in the human diet. All glucosinolates share a common structure containing a β–D-thioglucose group, a sulfonated oxime moiety and a side chain R (R= alkyl, alkenyl, arylalkyl, alkylthioalkyl, β-hydroxyalkyl or indolylmethyl).

The enzyme myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1) catalyses the hydrolysis of glucosinolates. Myrosinase is found in the same plant cells but in a separate compartment. Food processing, cutting, and especially chewing mix the myrosinase and glucosinolates and hydrolysis takes place. Myrosinase activity is also present in some intestinal microflora. The myrosinase-catalyzed hydrolysis of the glucosinolate *S*-sugar bond leads to an unstable thiohydroxamic acid, which undergoes a Lossen rearrangement, yielding the ITC. Depending on the R group and the conditions, other products such as nitriles, thiocyanates, or alcohols may also form.

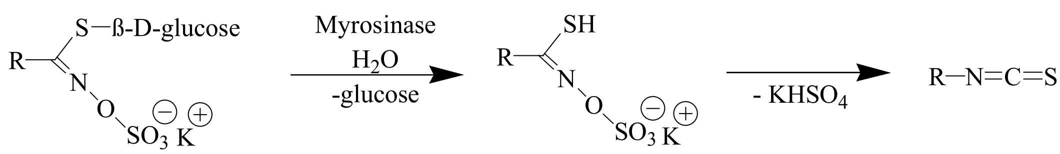


Figure 4: Conversion of glucosinolates to ITCs

1.6 Animal models for carcinogenesis

The tobacco-specific *N*-nitrosamine NNK and the polycyclic aromatic hydrocarbon B[*a*]P are believed to be two of the most important carcinogens in tobacco smoke. Therefore, NNK and B[*a*]P are widely used as model carcinogens (Table 2, reviewed in (31)) for the development of chemopreventive agents (reviewed in (130)).

A wide variety of compounds or mixtures has been tested for their potencies to prevent NNK-induced lung tumorigenesis (reviewed in (31)). For example; while some ITCs (135), green and black tea (136), caffeine (137), sulindac (138), ibuprofen (139) and aspirin (140) had preventive effects, oltipraz (141), β-carotene and retinol (142) and some ITCs (135) had no effect. Among the compounds tested so far, ITC administrated before NNK treatment were the most potent inhibitors. Studies showed that phenethyl isothiocyanate (PEITC) significantly reduced lung tumor incidence and multiplicity in A/J mice and F-344 rats treated with NNK (143), but not in animals treated with B[*a*]P (144). Benzyl isothiocyanate (BITC), on the other hand, reduced the formation of lung tumors in A/J mice treated with B[*a*]P (145), but had no effect on NNK-induced lung tumorigenesis (Table 2) (146),(147).

While early studies mostly used single carcinogen treatments, our group recently established an A/J mouse lung tumor model using NNK plus B[*a*]P as carcinogens (148). In the initial model, mice were treated with a mixture of NNK plus B[*a*]P (3 µmol each) by gavage once weekly for eight weeks. Nineteen weeks after the last carcinogen treatment, the mice were sacrificed and lung tumors counted. This protocol typically results in 20-25 lung tumors per mouse, a number that can be readily increased or decreased depending on study purposes. Using this model, the efficacy of mixtures of different chemopreventive compounds were evaluated (149,150). ITCs administrated by gavage 2 h prior to the carcinogens [BITC (9 µmol) and PEITC (12 µmol), or 12 µmol of each], modestly but significantly inhibited lung tumor multiplicity by 30-35%, while lower doses (9 or 6 µmol of each) were ineffective, as were 6 µmol of either compound given alone. The administration of the chemopreventive agents in the diet (PEITC dose 3 µmol/g plus BITC dose 1 µmol/g diet) starting 1 week prior to the first carcinogen treatment significantly inhibited tumor multiplicity by 40%. This model has been successfully used to examine the effects of a variety of other chemopreventive agents (149,151,152).

In a widely used rat esophagus tumor model, several asymmetric nitrosamines were found to be strong inducers of esophageal tumors (26). Prominent among these is *N*-nitrosomethylbenzylalamine (NMBA), which is widely used as a model carcinogen. While NMBA is an excellent model carcinogen, its relevance to the human situation remains to be established. The tobacco-specific *N*-nitrosonornicotine (NNN), on the other hand, is a well-established esophageal carcinogen in rats, and human exposure through tobacco usage is well known (153-155). Stoner et al. revealed that chronic exposure to NNN via drinking water produces tumor incidences higher than expected from previous reports, and PPITC significantly inhibited the NNN-induced esophageal tumorigenesis (156). This is another suitable model to investigate the mechanism by which ITCs inhibit tumor development.

Table 2: Effects of BITC and PEITC on NNK and B[*a*]P-induce tumors in rodents.[a]

group	Carcinogen	Lung tumor multiplicity	Lung tumor incidence	Reference
A/J mouse[b]				Morse et al. 1989
none	NNK	9.2	100%	
BITC	NNK	10.4	100%	
PEITC	NNK	3.3	93%	
F344 rats[c]				Hecht et al. 1996
none	NNK		70%	(143)
PEITC	NNK		5%	
A/J mouse[d]				Wattenberg 1987
none	B[*a*]P	15.5		(145)
BITC (1 mg)	B[*a*]P	7.4		
BITC (2.5 mg)	B[*a*]P	3.9		
A/J mouse[e]				Lin et al. 1993
none	B[*a*]P	4.8	95%	(144)
PEITC	B[*a*]P	4.0	90%	
BITC	B[*a*]P	2.6	80%	
A/J mouse[f]				Hecht et al. 2000
none	NNK plus B[*a*]P	25.1	100%	(135)
PEITC	NNK plus B[*a*]P	14.0	100%	
BITC	NNK plus B[*a*]P	26.6	100%	
PEITC plus BITC	NNK plus B[*a*]P	16.8	100%	
F344 rats[g]				Stoner et al.
None	NNN	1.57	71.4%	(72)
PPITC	NNN	0.06	2.9%	

[a] Shown is a selection. For a comprehensive review of ITC see Hecht 2002 (130).
[b] ITC (5 µmol) were given daily by gavage for four days 2 h prior to intarperitoneal (i.p.) administration of NNK (10 µmol).
[c] PEITC was given in the diet (3 µmol/ g diet), NNK (2 ppm) was given in drinking water.
[d] ITCs were given by gavage 15 min prior to administration of B[*a*]P (6.7 µmol).
[e] ITCs were given by gavage (6.7 µmol) 15 min prior to administration of B[*a*]P (7.9 µmol) and NNK (7.9 µmol) or B[*a*]P plus NNK (7.9 µmol each) once every other week for a total of three treatments.
[f] PEITC (3 µmol/g diet) and BITC 1 µmol/g diet) were given in the diet, NNK plus B[*a*]P (3 µmol each in 0.2 ml cottonseed oil) were given by gavage.
[g] NNN was given in the drinking water (2ppm) and PPITC was given in the diet (2.5 µmol/g).

1.7 Goals and hypothesis

Our long-term goal is to develop mixtures of chemopreventive agents as inhibitors of lung cancer induced by carcinogens in tobacco smoke. Therefore, understanding the molecular mechanism of cancer induction and prevention is essential (114,115). Research has demonstrated the importance of DNA adduct formation in NNK and B[*a*]P-induced lung carcinogenesis (40). Furthermore, it is well established that ITCs effectively inhibit tumor formation by NNK plus B[*a*]P (135). However, the effects of ITCs on DNA adducts from B[*a*]P plus NNK treatments have not been evaluated. It is also well known that NNN, a potent esophageal carcinogen, produces HPB-releasing DNA adducts (44,45,67), and ITCs are capable of inhibiting NNN-induced esophageal carcinogenesis (72).

We therefore hypothesize that the preventive effects of ITCs and their *N*-acetylcystein conjugates (BITC, PEITC, PPITC, PEITC-NAC, and PPITC-NAC) are due to inhibition of DNA adduct formation. These effects are expected regardless of tissue (lung or liver) or species (mice or rats). To study this hypothesis, the following goals were set:

1. To detect DNA adducts of NNK and B[*a*]P, methods will be established in our laboratory. We will focus on a GC-NICI-MS technique for the detection of NNK-derived adducts and on HPLC-FD and GC-NICI-MS techniques for the specific analysis of B[*a*]P. This method will then be applied to animal studies.
2. The established methods will then be applied to determine the effects of DNA adduct formation in A/J mouse lung when NNK and B[*a*]P are given alone or in combination. This will guarantee that the effects observed in the later studies are due to ITCs and are not results of NNK on B[*a*]P or *vice versa*.
3. Using conditions identical to those in the tumor inhibition studies (135), the effects of PEITC and BITC (administered by gavage or in the diet) on lung DNA adduct formation will be determined in mice treated with a mixture of NNK plus B[*a*]P. Herein, we will apply HPLC-FD for the analysis of B[*a*]P-tetraol-releasing DNA adducts, and HPB-releasing adducts of NNK will be analyzed by GC-NICI-MS. Co-workers will also determine the levels of O^6-mG by HPLC-FD. Altogether, these

results will determine to what extent ITCs affect the formation of the analyzed adducts.

4. To assess the effects of PEITC and BITC in other species, we will extend our research to F-344 rats using the same treatment protocol as applied in the mouse dietary experiment. Moreover, NNK and B[*a*]P adducts will be determined in lung and liver DNA as well as in globin. HPB-releasing and B[*a*]P-tetraol adducts in lung and liver DNA as well as from globin will be determined by GC-NICI-MS. This will clarify whether the mechanism of prevention by ITC of NNK-induced lung tumors is the same for mice and rats.
5. Our last goal will be to determine whether the inhibition of DNA adducts by ITC applies to other carcinogens. To achieve this, NNN will be used as a model carcinogen, and PEITC and PPITC as well as their NAC conjugates will be used as preventive agents. HPB-releasing DNA adducts in esophagus, the target tissue of NNN, and globin adducts will be analyzed in rats treated with NNN and PEITC and PPITC or their NAC conjugates. Treatments will be adopted from a protocol known to cause sufficient number of tumors, as well as tumor inhibition (72).

2 Materials and Methods

2.1 Enzymes and Chemicals

B[*a*]P, PEITC, BITC and [^{13}C]methyl iodide were obtained from Aldrich Chemical Co. (Milwaukee, WI). NNK was synthesized (157). B[*a*]P-tetraol standards were obtained from the National Cancer Institute Chemical Carcinogen Reference Standard Repository, Midwest Research Institute (Kansas City, MO). [$^{2}H_{8}$]B[*a*]P-tetraol was a generous gift from Assieh Melikian, American Health Foundation. PFBC was obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents and solvents were from Fischer Scientific (Hanover Park, Il). All reagents were OPTIMA or GC-Resolve grade. Coffee and cookies were purchased at the local grocery stores.

2.2 Animal experiments

Tissue samples were from four ongoing animal studies aimed at understanding molecular mechanisms of chemopreventive agents. All the animal handling and treatments at the University of Minnesota Cancer Center were done by P. Kenney in our laboratory (animal study #1- #3) or by laboratory personnel of our collaborator G. Stoner at the Ohio State University (animal study #4). Treatment procedures were as follows.

Arrival and general handling of the animals

Female A/J mice were obtained at age 5-6 weeks from Jackson Laboratories (BarHabor, ME). They were housed under standard conditions and maintained on an AIN-93G diet (Dyets, Bethlehem, PA). Male F-344 rats were obtained at age 8 weeks from Charles River, Wilmington, MA. They were housed under standard conditions and maintained on an NIH-07 diet (Dyets, Bethlehem, PA). After arrival, all animals were allowed to acclimate to the animal facility for two weeks. Animals were house under standard conditions (22 ± 2°C, 40 ± 10% relative humidity) either 5 mice or 2 rats per cage, respectively (158).

2.2.1 DNA adduct formation in lungs of A/J mice treated with B[*a*]P and NNK simultaneously.

In the first animal study, our goal was to determine whether formation of DNA adduct levels in the lung were different when B[*a*]P and NNK were given alone or simultaneously (see Table 3). One hundred and eight A/J mice, 5-6 weeks old, were divided into 4 treatment groups of 27 mice per group. All mice were treated weekly by gavage with either cottonseed oil (2 ml), NNK (3 µmol in 0.2 ml cottonseed oil), B[*a*]P (3 µmol in 0.2 ml cottonseed oil) or NNK plus B[*a*]P (3 µmol each in 0.2 ml cottonseed oil). After 1, 4 and 8 weeks, 9 mice from each group were sacrificed 24 h after the last carcinogen treatment. Lung tissues were harvested from each group and divided into 3 pools of 3 lungs each. The tissues were frozen in liquid nitrogen and stored at -80°C until DNA isolation.

Table 3: Experimental design for animal study #1

Group[a]	Number	Carcinogens
1	27	3µmol NNK in 0.2 ml cottonseed oil
2	27	3µmol B[*a*]P in 0.2 ml cottonseed oil
3	27	3µmol NNK plus 3µmol B[*a*]P in 0.2 ml cottonseed oil
4	27	0.2 ml cottonseed oil

[a] Nine mice from each group were sacrificed 24h after 1, 4 or 8 weekly carcinogen treatments, by gavage.

2.2.2 Effects of PEITC and BITC given by gavage or in the diet on adduct formation by NNK plus B[*a*]P in A/J mouse lung.

In the second animal study, we analyzed the effects of PEITC, BITC and PEITC plus BITC given by gavage or in the diet on the formation of DNA adducts derived from NNK plus B[*a*]P in the lungs of A/J mice (see Figure 5, Table 4). A total of 378 A/J mice, 5-6 weeks old, were divided into 9 groups of 42 mice each. All mice were treated with NNK plus B[*a*]P by gavage (3 µmol each in 0.2 ml cottonseed oil). From each group 18 mice were given a single dose of NNK plus B[*a*]P and 6 mice were sacrificed 4, 24 and 120 h later. Twelve mice per group were given 4 weekly treatments of NNK plus B[*a*]P and 6 mice were sacrificed 24 and 120 h later, and 12 mice were given 8 weekly treatments of NNK plus B[*a*]P and 6 mice were sacrificed 24 and 120 h later. At sacrifice, lung tissues were harvested and 3 pools of 2 tissues each were stored at –80°C until DNA isolation. Of the nine groups, six treatments were gavage administration and three were dietary. In the gavage experiments, treatments were as follows: group 1) 0.2 ml cottonseed oil 2h prior to carcinogen treatment; group 2) 6 µmol PEITC plus 3 µmol BITC in 0.2 ml cottonseed oil; group 3) 6 µmol PEITC plus 6 µmol BITC in 0.2 ml cottonseed oil; group 4) 9 µmol PEITC plus 9 µmol BITC in 0.2 ml cottonseed oil; group 5) 12 µmol PEITC plus 9 µmol BITC in 0.2 ml cottonseed oil; and group 6) 12 PEITC plus 12 µmol BITC in 0.2 ml cottonseed oil. In the dietary experiment, the ITC administration started one week prior to the carcinogens. The dietary additions were as follows: group 7) none; group 8) 3 µmol PEITC/g diet; and group 9) 3 µmol PEITC plus 1 µmol BITC/ g diet. The diets were prepared monthly and stored at 4 °C.

Table 4: Experimental design for animal study #2

Group[a]	Number	Carcinogens[b]	Isothiocyanates[c]
1	42	NNK plus B[*a*]P	None (cotton seed only)
2	42	NNK plus B[*a*]P	6 µmol PEITC plus 3 µmol BITC
3	42	NNK plus B[*a*]P	6 µmol PEITC plus 6 µmol BITC
4	42	NNK plus B[*a*]P	9 µmol PEITC plus 9 µmol BITC
5	42	NNK plus B[*a*]P	12 µmol PEITC plus 9 µmol BITC
6	42	NNK plus B[*a*]P	12 µmol PEITC plus 12 µmol BITC
7	42	NNK plus B[*a*]P	None
8	42	NNK plus B[*a*]P	3 µmol PEITC/g diet
9	42	NNK plus B[*a*]P	3 µmol PEITC plus 1 µmol BITC/g diet

[a] Each group consisted of 7 subgroups of 6 mice each differing in duration of carcinogen treatments, once a week for 1, 4 or 8 weeks and time of sacrifices at 4, 24 or 120 h after the last carcinogen treatment (see text for details).
[b] NNK plus B[*a*]P (3 µmol each in 0.2 ml cotton seed oil) were given by gavage once a week.
[c] ITC were given by gavage (groups 1-6) 2h prior to the carcinogen administration or in the diet (groups 7-9) starting 1 week prior the carcinogen administration at dosages indicated.

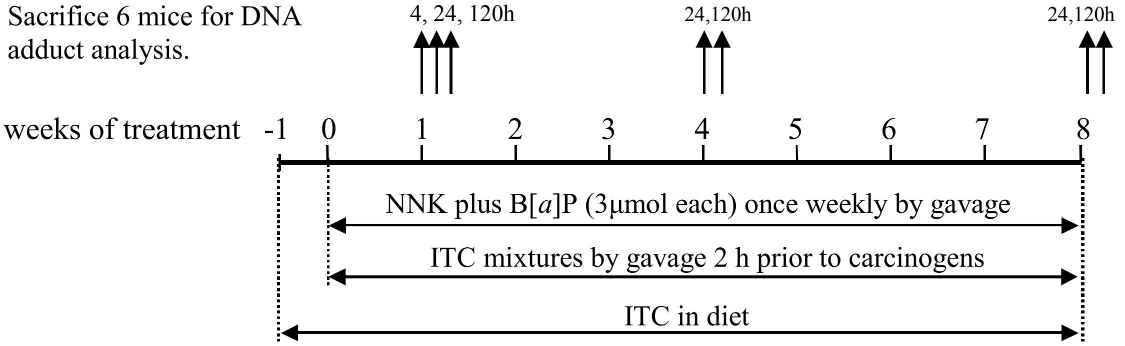


Figure 5: Scheme of animal study #2

2.2.3 Effects of PEITC and BITC on adduct formation by B[*a*]P plus NNK in F-344 rats.

In the third animal study, we studied the effects of PEITC, BITC and a mixture of PEITC and BITC on globin and DNA adduct formation from chronic exposure to NNK and B[*a*]P. F-344 rats were 10 weeks of age at the beginning of the carcinogen treatment. Forty-eight rats were divided into 4 groups (see Figure 6, Table 5). All rats were given NNK in the drinking water (2 ppm) and B[*a*]P in the diet (2 ppm, 2 mg/kg diet) *ad libitum*. The groups receiving dietary ITC treatment were placed on a special diet containing ITC one week prior to the carcinogen treatment. Dietary additions were as follows: group 1) none; group 2) 1 µmol BITC /g diet group; 3) 3 µmol PEITC /g diet; group 4) 1 µmol BITC plus 3 µmol PEITC µmol/g diet. Starting 2 weeks after the first carcinogen treatment, every two weeks for 16 weeks, 4 rats from each group were randomly selected, and 0.5-1.0 ml blood was withdrawn from the orbital sinus. Blood was collected in tubes containing EDTA. The red blood cells (RBC) were pelleted by centrifugation, washed three times with 1 volume saline (0.9% NaCl) and stored at -80°C. Eight weeks after the first carcinogen administration, 6 rats were randomly selected and sacrificed. Sixteen weeks after the first carcinogen administration, the remaining rats were sacrificed. At the final sacrifice, approximately 5 ml of blood was drawn by cardiac puncture, and lung and liver tissues were harvested and stored at –80°C (Figure 6).

Table 5: Experimental design for animal study #3

Group	Number	Isothiocyanates[a]	Carcinogens[b]
1	12	None	NNK plus B[*a*]P
2	12	PEITC/diet	NNK plus B[*a*]P
3	12	BITC/diet	NNK plus B[*a*]P
4	12	PEICT plus BITC/diet	NNK plus B[*a*]P

[a] PEITC dose = 3µmol/g diet; BITC dose = 1 µmol/g diet).
[b] NNK dose = 2 ppm in the drinking water, B[*a*]P dose = 2ppm in diet.

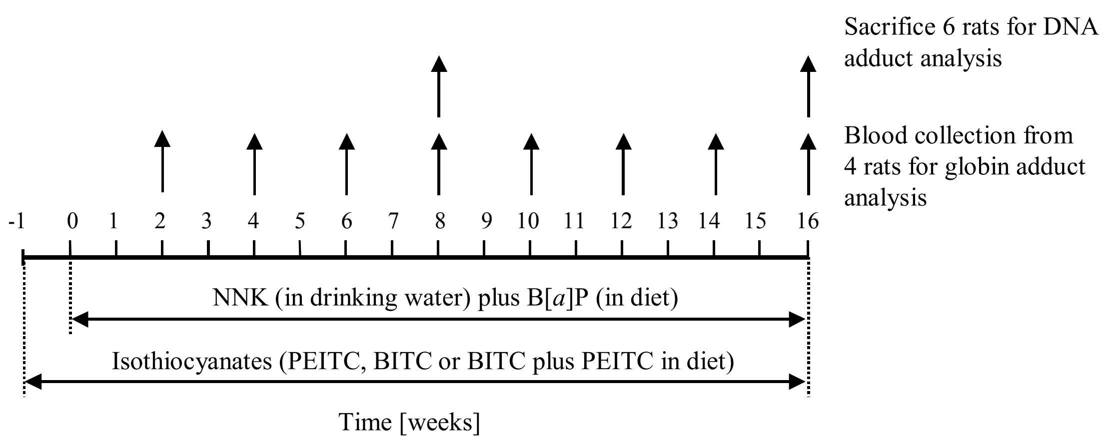


Figure 6: Scheme of animal study #3

2.2.4 Effects of PEITC and PPITC as well as their NAC conjugates on adduct formation by NNN in esophageal DNA and Hb of F-344 rats.

In collaboration with G. Stoner (Ohio State University), we compared the efficiencies of PEITC, PPITC and their *N*-acetylcysteine (NAC) conjugates on NNN-induced esophageal tumorigenesis using HPB-releasing Hb and DNA adducts as short-term biomarkers. F-344 rats, 5 weeks old, were divided into 6 treatment groups of 45 animals each. Group 1 was a control, neither treated with NNN nor with any ITCs. In Groups 2 – 6, NNN was given in drinking water (5 ppm) and the rats were placed on a diet containing 1.0 µmol PEITC, PEITC-NAC, PPITC or PPITC–NAC per g diet, respectively (Table 6). Blood was drawn by cardiac puncture prior to sacrifice and separated into plasma, WBC and RBC. At 4, 10 and 16 weeks, 15 rats from each group were sacrificed and the esophagi and RBC pellets were prepared and shipped on dry ice to our laboratory and stored at -80˚C until use.

Table 6: Experimental design for animal study #4

Group[a]	Number	Carcinogen[b]	Isothiocyanates[a]
1	45	None	None
2	45	NNN	None
3	45	NNN	PEITC
4	45	NNN	PEITC-NAC
5	45	NNN	PPITC
6	45	NNN	PPITC-NAC

[a] NNN dose = 5ppm in drinking water.
[b] ITCs or conjugates dose = 1.0 µmol/g diet

2.3 Collection and handling of specimens

2.3.1 Preparation of hemoglobin from RBC

Hemoglobin was isolated from RBC via a standard method. In brief, the RBC samples were lysed by adding 1 volume H_2O and kept on ice for 10 min, followed by adding 1 volume 0.67 M NaH_2PO_4, pH 6.5, to make a final concentration of 0.2 M. Samples were than transferred to 50 ml centrifuge tubes (Nalgene 3119-0050) and centrifuged at 25,000g for 30 min at 4°C (Sorvall centrifuge). The Hb solution was transferred into a dialysis tube (Spectra/Por, MW cutoff 15.000) and the cell debris (pellet) was discarded. Hb solutions were dialyzed at 4°C against 20 volumes H_2O for 3h. Water was changed every hour. Dialyzed Hb solution was transferred to 50 disposable centrifuge tubes and stored at –20°C.

2.3.2 Precipitation of globin

To precipitate the globin, 2 ml of Hb solution was added dropwise to 40 ml ice-cold 1% HCl/acetone. The supernatant was discarded and the globin was washed twice with 100% acetone. Globin was dissolved in 2 ml H_2O and the precipitation was repeated twice. The washed globin was dried in a GC oven at 50°C over night and stored at –80ºC.

2.4 DNA Isolation

Solutions and mixtures

The homogenization buffer consisted of 15 mM Na-citrate, 150 mM NaCl, pH 7. The digest buffer was 1.5 ml of 15 mM Na-citrate pH 7. Solutions of 10% SDS and 100 mM EDTA were prepared. All buffers were stored in a cold room (4°C) except for the 10% SDS solution, which was stored at room temperature. The proteinase K stock solution (Sigma P-4789) was: 1U/ µl in 10 mM Tris-HCl buffer, pH7. The RNase A (Sigma R-5215) stock solution was: 0.5U/ µl 10mM Tris-HCl-buffer, pH7. The RNase T1 (Sigma R-8251) stock solution was: 50U/ µl. All protein stock solutions were stored as 100 µl aliquots at -20°C.

DNA isolation from mouse tissues

All of the following steps were carried out on ice, until proteins were added. The tissue was thawed on filter paper and weighed. Approximately 200 mg of tissue in 3 ml homogenizing buffer (15 mM Na-citrate, 150 mM NaCl, pH 7) was homogenized using a glass homogenizer (pistel B) and centrifuged at 10,000 g for 15 min (70.1 Ti rotor at 10000 rpm). The supernatant was discarded carefully and the pellet was resuspended in the digest buffer (1.5 ml of 15 mM Na-citrate pH 7). This was followed by adding 600 µl 2 M NaCl, 33 µl (10% SDS) and 33 µl 100 mM EDTA. Ten units of proteinase K were added and the samples were incubated for 40 min at room temperature. After the proteinase K treatment, the solutions were extracted 3 times with 3 ml chloroform/ isoamyl alcohol (24:1) and centrifuged at 14,000g for 15 min at 4˚C. The aqueous layer was transferred to a new tube and the samples were treated with 10 units of RNase A and 750 units RNase T1 for 40 min at room temperature. Ten units proteinase K were added and the samples were incubated for an additional 40 min at room temperature. Afterwards, the samples were chilled on ice for 10 min and extracted 3 times with 1 volume chloroform/isoamyl alcohol (24:1) as described above. The DNA was precipitated with ice cold 100% EtOH. The DNA pellet was then rinsed once with 70% and twice with 100% EtOH (1 ml each), dried under a gentle steam of nitrogen, and stored at –80°C.

DNA isolation from rat tissues Progene® Isolation kit

DNA was isolated using the Progene® DNA isolation kit (Gentra, Minneapolis, MN) according to the manufacturers description. In brief, tissues were honogenzed in 3 ml of 'Cell Lysis Solution' using 10-50 strokes in glass homogenizer. To digest the protein, 15 µl 'Proteinase K Solution' (20 mg/ml) was added, and samples were incubated at 55˚C for 5 h. Then RNase A Solution was added and samples were incubated at 37˚C for 1 h. Proteins were precipitated by adding 'Protein Precipitation Solution' followed by centrifugation at 2,000 g for 10 min. The pellet was discarded. DNA was precipitated by slowly adding 3 ml isopropanol. DNA was then transferred with a Pasteur pipette to 4 ml silanized vials. DNA was rinsed once with 70% and twice with 100% EtOH (1 ml each), dried under a gentle steam of nitrogen and stored at -20˚C until adduct analysis.

2.5 Synthesis of standards

2.5.1 Synthesis of BPDE-DNA and [$^2H_8$]BPDE-DNA

Ten mg calf thymus DNA (*c.t.*DNA) in 10 mL 50mM Tris buffer pH 7 was reacted with 1 mg BPDE or [$^2H_8$]BPDE in 500 µL THF for 8h at 37ºC. The adducted DNA was extracted 5 times with ethyl acetate to remove unreacted B[*a*]P metabolites. The BPDE-DNA was precipitated with NaCl and EtOH. Therefor 0.1 volume of 5 M NaCl was added and 2 volume of 100% EtOH were added slowly until DNA precipitated. The DNA was further washed once with 70% then with 100% EtOH until the EtOH rinse had no detectable amount of unbound B[*a*]P-tetraols as determined by HPLC-FD using an isocratic system as described below (see section 2.6).

2.5.2 Synthesis of BPDE-3'-dGMP standards

Solutions and mixtures

The following solutions were prepared: 10 × digest buffer: 100 mM sodium succinate pH 6.0, 50 mM CaCl, pH 6.0. MN/PDE II mix: 1mg micrococcal nuclease *S*7 (MN) (Boehringer Mannheim Corp., Indianapolis, IN) and 1mg phosphodiesterase II (PDE II) (Sigma, St Louis, US) were dissolved in 1ml digest buffer. The final concentration was 1 µg/µl each MN and PDE II.

Enzyme hydrolysis of synthetic BPDE-DNA

The BPDE-DNA or [$^2H_8$]BPDE -DNA was digested essentially as described by Gupta (94). The DNA was digested with MN and PDE II to give the 3'-monophosphates. In brief, 2.5 mg BPDE-DNA was dissolved in 4.0 ml and 0.5 ml 10 × digest buffer and 0.5 ml MN/PDE II mix (500 µg/ µl of each enzyme) was added and the digest was incubated for 5 h at 37˚C.

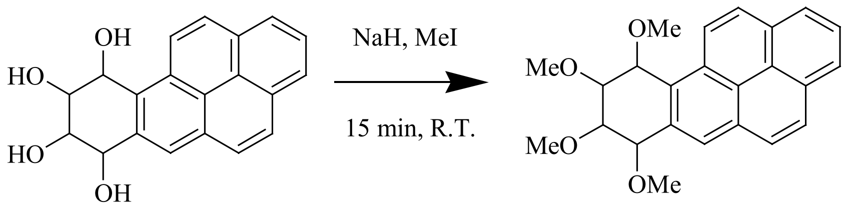
Solid-phase extraction (Sep-Pak®)

After the enzyme digestion, the BPDE-3'-dGMP was separated from unmodified nucleotides and enzymes by solid-phase extraction. A 500 mg C_{18} Sep-Pak® cartridge (Waters, Milford, MA) was conditioned with 10 mL of 100% methanol and 5 mL H_2O, and 5 mL digest buffer prior to the sample loading. The entire digest solution was loaded onto the cartridge. The cartridge was washed with 5 mL of digest buffer and 5 ml of 20% methanol/water. The BPDE-3'-dGMP adduct was eluted with 5 mL of 100% methanol (Fischer, OPITMA). The solvent was removed under a gentle steam of nitrogen. BPDE-3'-dGMP was further purified by HPLC. The conditions were as follows: C_{18} reverse phase column (Beckmann, Ultrasphere ODS $C_{18,}$ 5µm, 4.6 × 250 mm) was operated with a gradient program of 100 % 50 mM KH_2PO_4 to 60 % MeOH in 60 min at a flow rate of 1 ml/ min. The eluting BPDE-3'-dGMPs were detected with an online photodiode array (PDA, Waters) and/or fluorescence detector (FD, Shimadzu). The BPDE-3'-dGMP fraction was collected and buffer constituents were removed by solid- phase extraction, as described above, using a 5 mg C_{18} Sep-Pak® cartridge. The BPDE-3'-dGMP standards were than further characterized by LC-ESI-MS and UV (200-400 nm) and quantified using the UV extinction coefficient $\varepsilon_{o(\lambda=279)}$= 41,000 (159).

2.5.3 Synthesis of B[*a*]P TMEs

The four B[*a*]P-TME isomer standards were synthesized using the four corresponding B[*a*]P-tetraol isomers as starting material. One µg B[*a*]P-tetraol in a 4 ml silane treated vial was dissolved in 400 µl DMSO. A magnetic miniature stirrer and approximately 2 mg of NaH were added and samples were stirred for 2 min. The reaction was started by adding 100 µl methyl iodide (MeI) and the reaction was allowed to proceed for 15 min with stirring at room temperature (Figure 7). The reaction was quenched by adding 500 µl H_2O. The B[*a*]P-TME were extracted 3 times with 1 ml benzene. The organic solvent was evaporated and the B[*a*]P-TMEs were further purified by reverse phase HPLC. The HPLC conditions were as follows: a C_{18} reverse-phase column (Beckman, Ultrasphere ODS, C_{18} 5 µ, 4.6 mm × 250 mm) was operated with an isocratic flow of 75% methanol/H_2O at a flow rate of 1 ml/min coupled with a photodiode array (PDA, Waters) and/or fluorescence detector (FD, Shimadzu). The

B[*a*]P-TMEs were dissolved in 50 µL MeOH and the entire sample was injected. The fractions were collected by time starting 1 min before the elution of *trans/anti* B[*a*]P TME (as determined before each analysis, 27-29 min), until 1 min after. The solvents were removed using a SpeedVac. The B[*a*]P-TME standards were then further characterized by GC-NICI-MS and UV (200-400 nm) and quantified using the UV extinction coefficient $\varepsilon_{o(\lambda=248)}$= 84,700. *trans/anti* [$^{13}CH_3$]$_4$ B[*a*]P-TME was synthesized using [^{13}C]-MeI. This was used as injection standards as well as a retention time marker.



trans/anti B[*a*]P-tetraol *trans/anti* B[*a*]P-TME

Figure 7: Scheme of derivatization

2.6 HPLC-FD analysis for B[*a*]P-tetraol–releasing DNA adducts

The method is a modification of that described by Alexandrov *et al.* 1992 and Rojas *et al*. 1994 (98,108) (Figure 8). The isolated dry DNA was transferred to a 4-ml silanized vial. The DNA sample was washed twice using 200 µl 100% EtOH and the EtOH rinse was checked for unbound B[*a*]P-tetraols by HPLC-FD. The HPLC conditions were as follows: a C_{18} reverse phase column (Beckman, Ultrasphere ODS, C_{18} 5 µ, 4.6 mm × 250 mm) was operated with an isocratic flow of 55% methanol at a flow of 1 ml/min. The retention times of the different B[*a*]P-tetraol isomers were determined with synthetic standards. The fluorescence-free DNA (100-500 µg) was dissolved in 610 µl H_2O. Ten µl of this solution was used for determination of DNA concentration and purity by UV. B[*a*]P-tetraols were released from the DNA solution under mild acid hydrolysis (0.1 N HCl, 4 h at 80˚C). The entire sample was injected on a gradient HPLC-FD system. The HPLC conditions were as follows: The B[*a*]P-tetraols were concentrated on a pre-column (Beckman Ultrasphere ODS C_{18}, 5 µm, 45 mm x 4.6 mm) with 35% methanol/H_2O isocratic flow at a rate of 1.0 ml/min. After 10 min, the flow was switched with a multi position valve (Valco instruments EC10W) to an analytical column (Beckman, Ultrasphere ODS C_{18} 5 µm, 4.6 mm x 250 mm). The B[*a*]P-tetraols were eluted with a gradient of 35% methanol/H_2O to 100% methanol in 30 min. The retention times of the different B[*a*]P-tetraol isomers were determined with synthetic standards. A Shimadzu RF-10Axl fluorescence detector was used for both HPLC-FD systems. The detector settings were as follows: gain 1, range 1, sense 2, Ex = 344 and Em = 398.

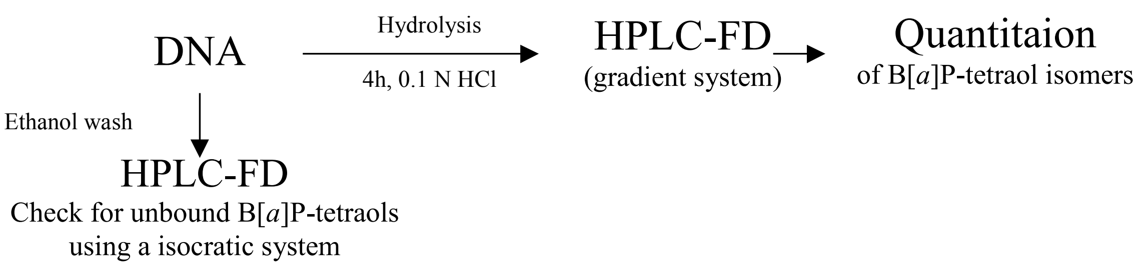


Figure 8: Outline B[*a*]P-tetraol HPLC-FD assay.

2.7 GC-NICI-MS analysis of B[*a*]P-tetraol–releasing DNA and globin adducts

The procedure is identical to the one described by Melikian et al. (110,160) but using a simplified permethylation method (161) (Figure 9).

Hydrolysis of DNA and globin. B[*a*]P-tetraols were released from DNA by mild acid hydrolysis. In brief, approximately 100 µg DNA was dissolved in 600 µl H_2O. Ten µl were used to determine the concentration by UV. Two pmol *trans/anti* [$^2H_8$]B[*a*]P-tetraol were added as internal standard. Samples were hydrolyzed under mild acid conditions (0.1 N HCl, 4 h at 80˚C). To release of B[*a*]P-tetraols from globin, 50 mg rat globin were dissolved in 3 ml H_2O. The globin solutions were transferred into an 8 ml vacuum hydrolysis vial and 2 pmol synthetic *trans/anti* [$^2H_8$]B[*a*]P-tetraol were added as internal standard. Adducts were released under mild acid hydrolysis (0.1 N HCl, 3h, 80˚C under vacuum). After hydrolysis, the globin was precipitated by neutralization with 0.4 N NaOH. The released B[*a*]P-tetraols were extracted 5 times with one volume ethyl acetate and the organic layer was removed with a SpeedVac concentrator and stored at -20°C.

HPLC Clean up #1. The released B[*a*]P-tetraols were further purified by reverse phase HPLC. The HPLC conditions were as follows: C_{18} column (Beckmann, Ultrasphere ODS, C_{18} 5 µ, 4.6 mm × 250 mm) was operated isocratically at 20% MeOH/H_2O for 10 min at a flow rate of 1 ml/min. The solvent composition was then increased linearly to 55% MeOH in 5 min and held for 20 min. The samples from globin extracts were dissolved in 70 µl 100% MeOH, while for the DNA samples, the total hydrolysis solution was injected with a series 1100 autosampler (Agilent, Wilminton, DE). The retention time of the *trans/anti* B[*a*]P-tetraols were determine before the each set of samples. The *trans/anti* B[*a*]P-tetraol usually eluted between 27 and 29 min. Fractions containing the *trans/anti* B[*a*]P-tetraol and *trans/anti* [$^2H_8$]B[*a*]P-tetraol were collected starting from 1 min before until 4 min after the elution of *trans/anti* B[*a*]P-tetraols. Solvents were removed with a SpeedVac concentrator and samples were stored at –20˚C until derivatization.

Derivatization of trans/anti B[a]P-tetraols to trans/anti B[a]P-TME. The derivatization was carried out at room temperature. Samples were dissolved in 100 µl DMSO. A magnetic miniature stirrer and approximately 2 mg of NaH were added and samples were stirred for 2 min. The reaction was started by adding 50 µl methyl iodide (MeI) and the reaction was allowed to proceed for 15 min with stirring at room temperature. The reaction was quenched by adding 500 µl H_2O. Derivatized *trans/anti* B[*a*]P-TMEs were extracted with benzene (3 × 1 ml). The benzene was removed and the samples were stored at –20˚C.

HPLC Clean up #2. The B[*a*]P-TMEs were further purified by reverse phase HPLC. The HPLC conditions were as follows: a 4.6 × 250 mm Beckmann Ultrasphere ODS C_{18} column was operated with a gradient program of 70 % to 100 % MeOH in H_2O over 30 min at a flow rate of 1 ml/ min. Samples were dissolved in 50 µl of MeOH containing 50 ng of hexanophenone and octanophenone as retention time markers. The entire sample was injected with an Agilent (Wilmington, DE) series 1100 autosampler. Fractions between apices of the UV-markers were collected with a fraction collector. Solvents were removed with a SpeedVac concentrator and samples were transferred to 300 µl insert vials with 3 × 250 µl MeOH and stored at –20˚C until analysis.

GC-NICI-MS. For analysis, samples were dissolvedin 10 µl benzene containing 150 fmol *trans/anti* $[^{13}CH_3]_4$B[*a*]P-TMS as an injection standard to monitor the instrument performance. 5 µl were injected in splittless mode. The analysis of B[*a*]P-TMEs was performed on a Finnigan TSQ 7000 instrument (FinniganMAT/Thermoquest, San Jose, CA) interfaced with CTC A200SE autosampler (Leap technologies, Carrboro, NC) and a HP5890 series II gas chromatograph (Agilent, Wilmington, DE). A DB-17 MS (30 m, I.D. 0.25 mm, film 0.15 µm) capillary GC column (J&W Sientific) connected to a 2 m x 0.530 µm fused silica uncoated deactivated retention gap, was interfaced to the CI source operated in negative ion mode. The oven temperature was held at 60ºC for 1 min and then increased to 300ºC at rate of 20ºC/min. The temperature was held at 300°C for 15 min. The MS parameters were as follows: ion-source temperature, 150ºC; emission current, 700 µA; electron energy, 150eV. Ultra high-purity methane was the reagent gas. The molecular ions of *m/z* 376 (*trans/anti* B[*a*]P-TME, analyte), *m/z* 380 (*trans/anti*

[$^{13}C]_4$B[*a*]P-TME, injection standard) and *m/z* 384 (*trans/anti* [$^{2}H_8$]B[*a*]P-TME , internal standard) were monitored.

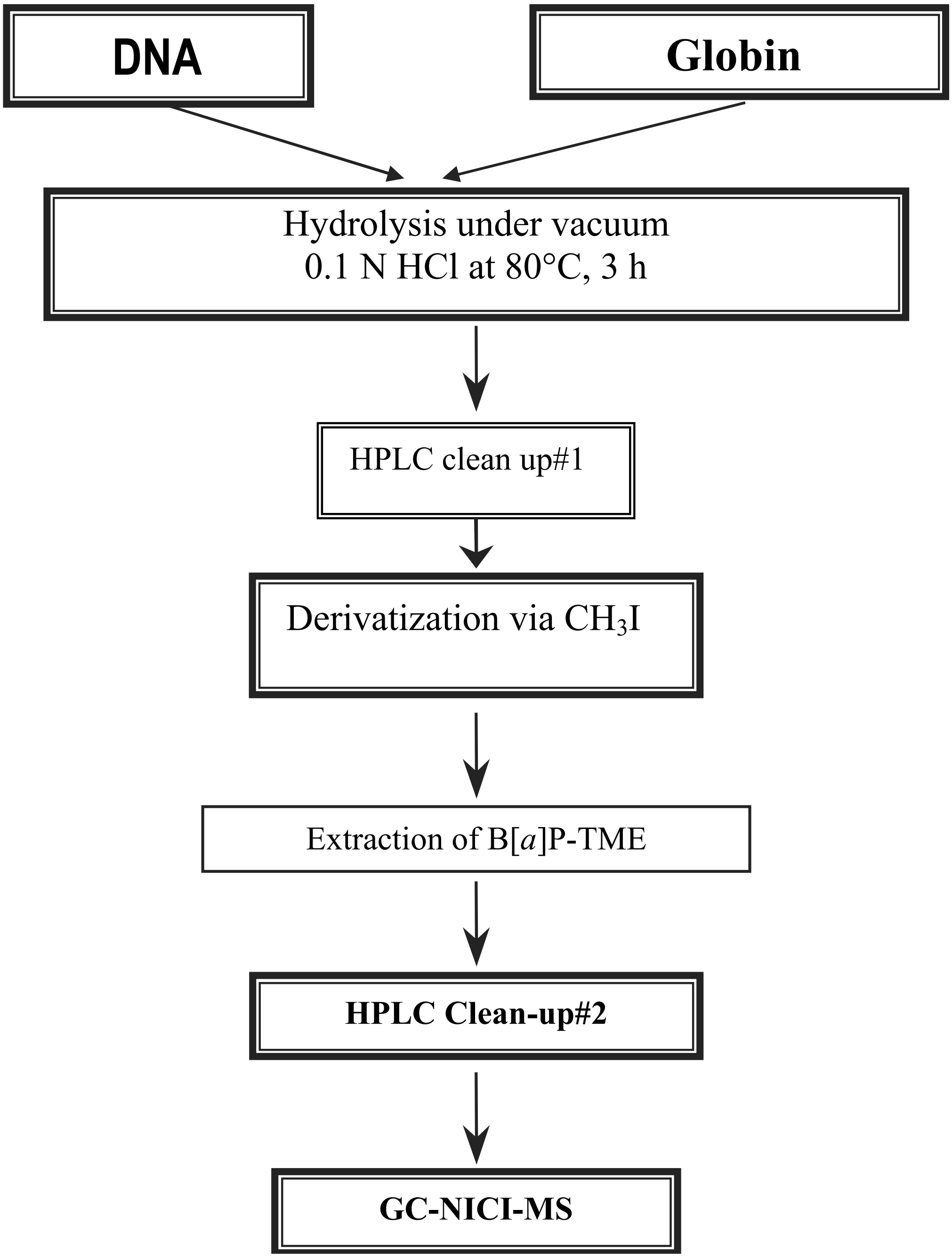


Figure 9: Outline of B[*a*]P-TME assay by GC-NICI-MS.

2.8 LC-MS analysis of BPDE-DNA from mouse liver

The DNA was digested to give the 3'-monophosphates using Micrococcal nuclease *S7* (Boehringer Mannheim) and spleen phosphodiesterase II (Sigma, St Loius, MO) as described by Gupta et al. 1996 (94). The DNA was dissolved in 210 µl H_2O and 10 µl was used for the determination of purity and concentration by UV spectrophotometry. For the analysis, approximately 300 µg DNA in 200 µl 1x digest was digested with 60 µl MN/PDE II mix for 5 h at 37˚C. The entire sample was injected onto the LC-MS with an on-column switching technique. The BPDE-3'-GMP was loaded onto a 1 x 150 mm Polaris 2000 column (trap column, Metachem) for 10 min at a flow rate of 200µl/min. The flow was then switched to a 1 x 150 mm Xtera column and the BPDE-3'-GMP was eluted with 45% MeOH/ 20 mM ammonium acetate buffer, pH 8.3, at 50 µl/ min. The MS was equipped with an electrospray ionization ion source and operated in negative ion single ion monitoring mode. The molecular ions *m/z* 648 (BPDE-3'-dGMP, analyte) and *m/z* 656 ($[^2H]_8$-3'-BPDE-dGMP, internal standard) were monitored.

2.9 GC-NICI-MS analysis of HPB-releasing DNA and globin adducts

The samples were analyzed in sets of 24, including 3 H_2O blanks (negative controls) and one H_2O blank spiked with 300 fmol HPB (positive control). Fifty pg (299 fmol) [4,4-$^2H_2$]HPB as internal standards was added to each sample.

Acid hydrolysis of DNA. The procedure was performed essentially as previously described (162,163)(Figure 10). Briefly, DNA was dissolved in H_2O and the DNA was analyzed by UV to insure sufficient purity and amount of DNA for analysis. The DNA was hydrolyzed by incubation at 80°C for 3 h at a final concentration of 0.8 N HCl in a total volume of 1.0 ml. Samples were extracted as described below.

Base treatment of hemoglobin. For the analysis of Hb adducts, 2.0 ml dialyzed Hb solution was used (see Section 2.3.1). The Hb concentration was determined by the Drabkin method (Sigma). For the base treatment, 4N NaOH was added to achieve a final concentration of 0.15 N NaOH. Samples were treated for 1 h at room temperature in a sonicator. Hemoglobin was then precipitated by neutralizing with 4N HCl. Fifty pg [$^2H_2$]-HPB 299 fmol was added as internal standard. The samples were vortexed for 1 min and centrifuged for 15 min (SpeedVac rotor). The supernatant was transferred to a new vial. The pH was adjusted to 2.0 ± 0.3 with 4 N HCl.

Extractions. The acidic aqueous sample solutions, from DNA or Hb, containing HPB and [$^2H_2$]HPB were extracted twice with equal volumes of CH_2Cl_2 . The Hb samples were additionally extracted twice with 1-volume hexanes. The aqueous layer was saved and the pH was adjusted to 7.0 with 1 N NaOH. The samples were then extracted three times with equal volumes of CH_2Cl_2. The organic layers were pooled and the solvent was removed with a SpeedVac concentrator.

Derivatization. The derivatization was carried out in a hexane solution of trimethylamine freshly prepared as follows: 240 mg trimethylamine hydrochloride (Sigma), in 20 ml 0.8 N NaOH were extracted with 20 ml hexane. The hexane layer was dried with approximately 2 g Na_2SO_4. Samples were dissolved in 0.5 ml CH_2Cl_2, and one ml of the hexanes/TMA solution was added. The reaction was started by adding 0.5 ml pentafluorobenzoyl chloride (PFBC) solution (20 µl PFBC in 10 ml CH_2Cl_2), and reaction was carried out for 1 h at room temperature. The solvents were removed using a SpeedVac concentrator. Unreacted PFBC was removed from the samples by reverse phase HPLC. The HPLC conditions were as follows: a C_{18} reverse phase column (Whatman Partisil 5 ODS 3, 4.6 mm x 12.5 cm) was operated with a gradient program of 35% MeOH/H_2O for 10 min followed by a linear gradient from 35% to 75% MeOH/H_2O in 15 min at 1 ml/min flow. For the HPLC clean up the samples were redissolved in 70 µl MeOH/THF (1:1) containing 25 µg/ml pentanophenone and hexanophenone (Sigma) as retention time markers. The entire sample was injected with a series 1100 autosampler (Agilent, Wilmington, DE). The fraction between the apices of the retention time marker compounds was collected with a fraction collector and dried in the SpeedVac concentrator. The residues were transferred to a silanized conical vial using 3 x 100µl THF and dried and stored at -20°C until GC-NICI-MS analysis.

GC-NICI-MS. The analysis of HPB-PFB was performed on a Finnigan TSQ 7000 instrument (FinniganMAT/Thermoquest, San Jose, CA) interfaced with a CTC A200SE autosampler (Leap technologies, Carrboro, NC) and a HP5890 series II gas chromatograph (Agilent Wilmington, DE). A DB-17 MS (30 m, I.D. 0.25 mm, film 0.15 µm) capillary GC column (J&W Scientific) connected to a 2 m x 0.530 µm fused silica uncoated deactivated retention gap, was interfaced to the CI source operated in negative ion mode. The GC oven program was as follows: 35˚C for 1 min, then 20˚C/ min to 150˚C, then 4 ˚C/ min to 205˚C, and finally held 20 min at a constant flow of 2.5 ml/ min helium. For GC-NICI-MS, samples were resuspended in 10 µl of toluene containing 6 fmol/ µl HPB-tetrafluorobenzoate (HPB-TFB) as external standard. The source temperature was 150˚C. The molecular ions of HBP-TFB, (injection standard, *m/z* 341), HPB-pentafluorobenzoate (HPB-PFB, analyte, *m/z* 359) and [$^2H_2$]HPB-PFB (internal standard, *m/z* 361) were monitored.

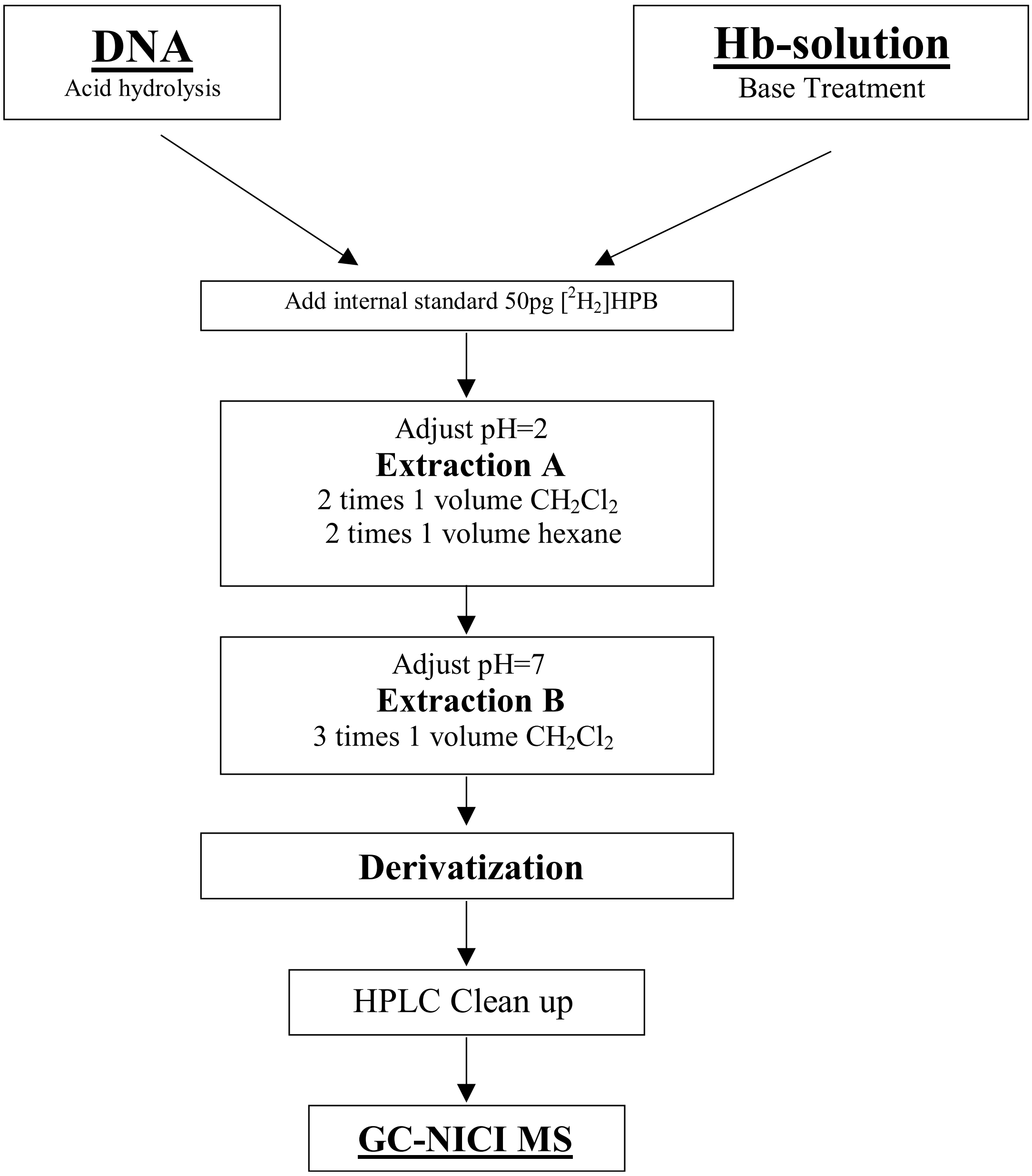


Figure 10: Outline of HPB assay

3 Results

3.1 Characterization of PAH standards

The different B[*a*]P-tetraol isomers were used as starting material for the synthesis and as reference standards for characterizing B[*a*]P-TME. The UV absorbance of the B[*a*]P-tetraol isomers and BPDE were identical, while those of pyrene were shifted by approximately 7~8 nm toward lower wavelengths (Figure 11). The conversion of the two hydroxyl groups to an epoxy group did not affect the UV absorbance. This confirms that the pyrene chromophore is the main absorbing part of the B[*a*]P-tetraols and BPDE. Hence, *anti* BPDE could be quantified using the extinction coefficient of *trans/anti* B[*a*]P-tetraol ($\varepsilon_{0\,(248)}$= 84700 or $\varepsilon_{0\,(279)}$ = 41000 (159)).

3.1.1 [$^{2}H_{8}$]BPDE-DNA standard

BPDE-DNA or [$^{2}H_{8}$]BPDE-DNA was prepared by reaction of calf thymus DNA (*c.t.*DNA) with BPDE or [$^{2}H_{8}$]BDE as described in Materials and Methods. Digestion to single nucleotides revealed that BPDE-N^2-dG was the major adduct formed in this reaction, as determined by HPLC-UV-FD of the digestion mixture after SepPak® cartridge clean up (Figure 12). The amount of BPDE-N^2-dG bound was determined by HPLC-FD, GC-MS and LC-MS (see chapter 3.2 on page 48).

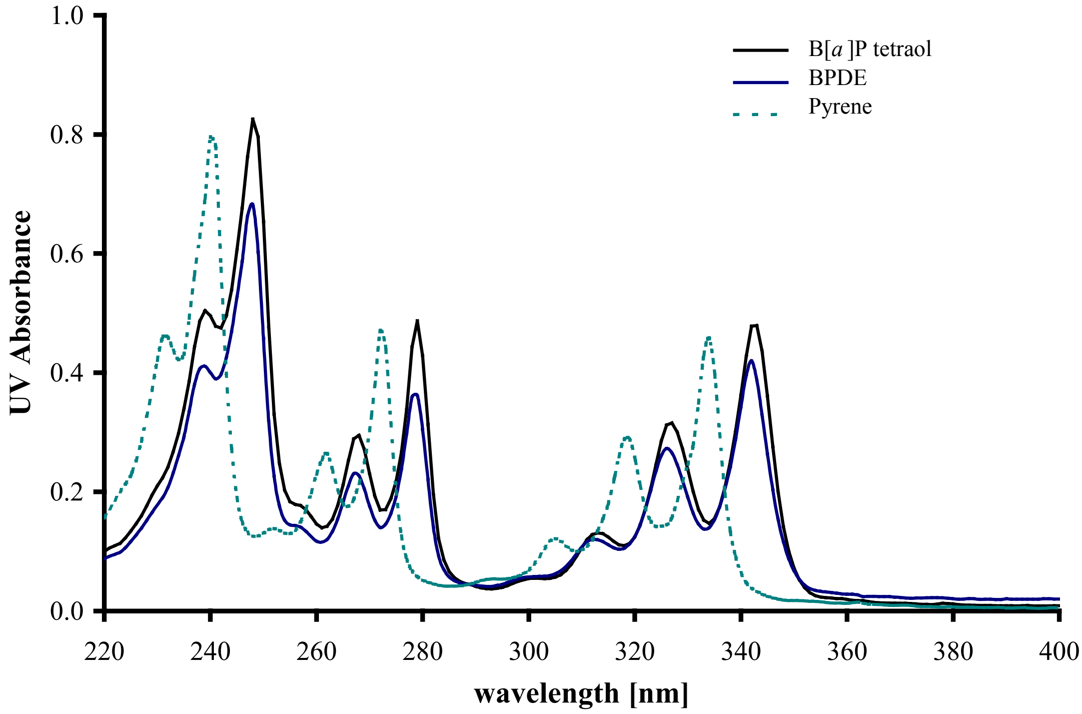


Figure 11: Comparison of UV scans of *trans/anti* B[*a*]P-tetraol, BPDE and pyrene. Concentrations were 9.6M, 8.9M, and 9.5M for *trans/anti* B[*a*]P-tetraol, BPDE and pyrene, respectively.

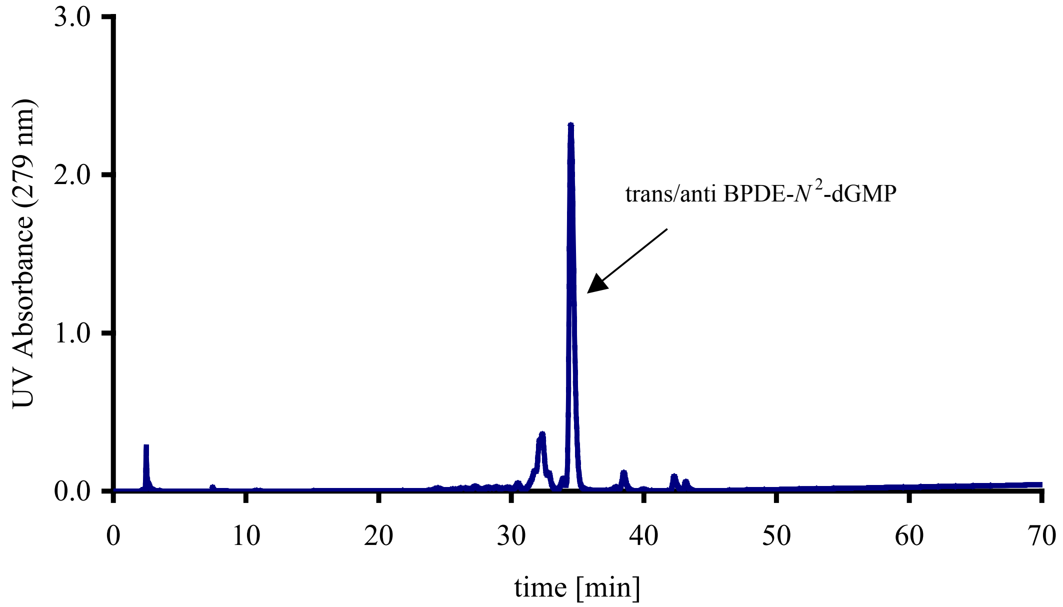


Figure 12: HPLC chromatogram of enzyme digested BPDE-DNA.

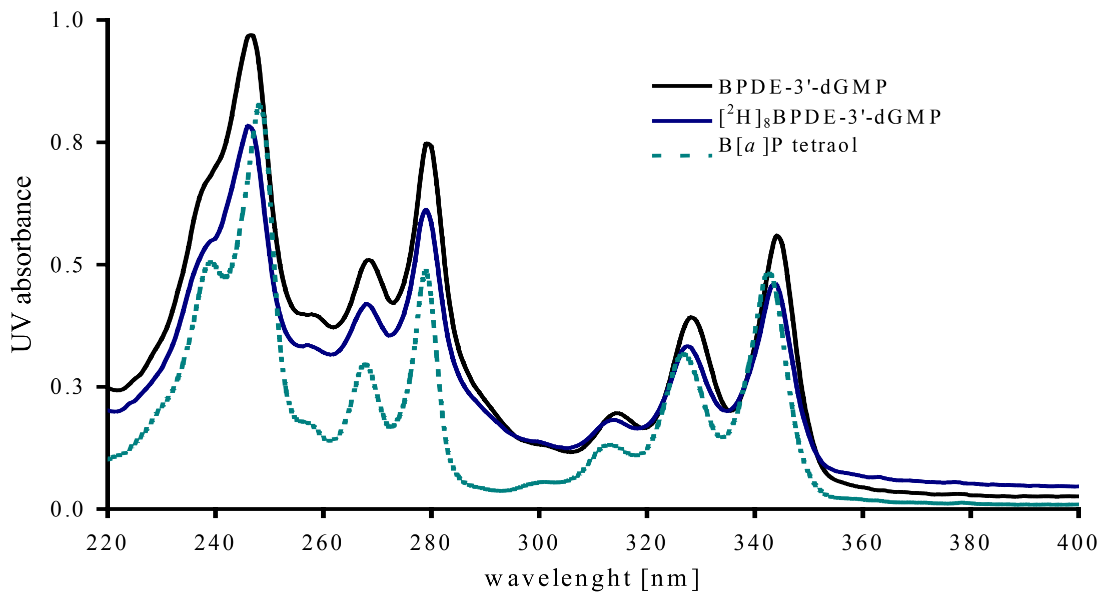


Figure 13: Comparison of UV scans of BPDE-3'-dGMP, [$^2H_8$]BPDE-3'-dGMP, and B[*a*]P-tetraol. Concentrations were 10 M, 9.3 M, and 9.6 M for BPDE-3'-dGMP, [$^2H_8$]BPDE-3'-dGMP, and *trans/anti* B[*a*]P-tetraol, respectively.

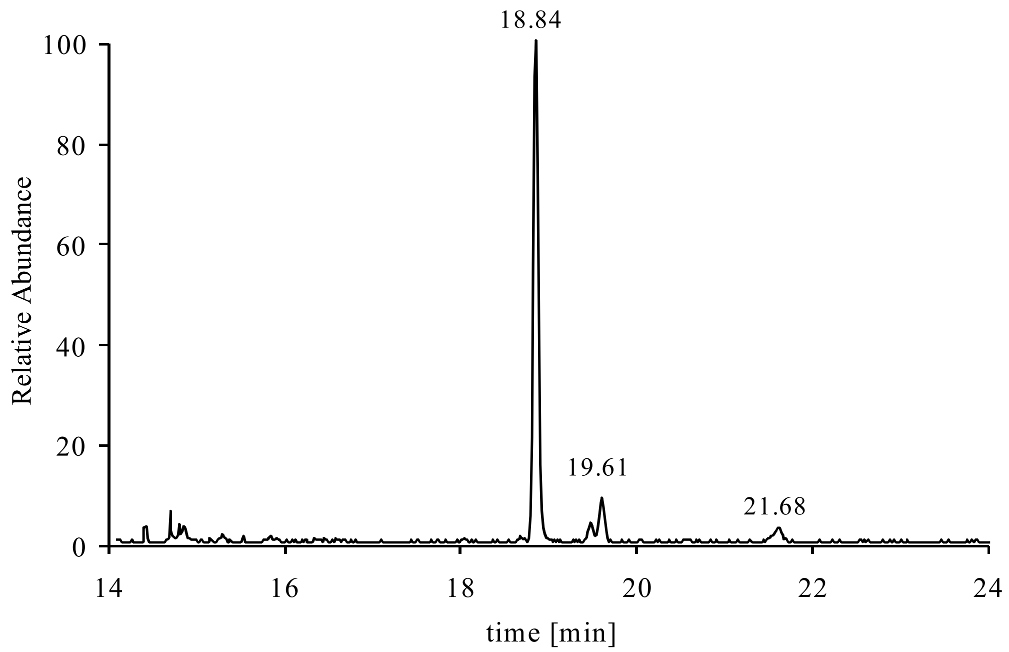
3.1.2 B[*a*]P-TME, $[^{2}H]_{8}$B[*a*]P-TME and $[^{13}CH_3]_4$B[*a*]P-TME

All four B[*a*]P-TME isomers were synthesized as described in Materials and Methods. The different B[*a*]P-TME isomers were characterized by their retention times on reverse-phase HPLC and GC-NICI-MS (Table 7). Purities were further confirmed by UV. The MS of the *trans/anti* B[*a*]P-TME peak is presented in Figure 14. The parent ion *m/z* 376 $[M]^-$ is clearly observed. The loss of methanol, forming a double bond, produces a fragment with *m/z* 344 $[M\text{-}32]^-$. This loses an additional methanol leading to fragment 312 $[M\text{-}64]^-$. The loss of two methoxy groups adjacent to each other produces only one double bond and generates the minor fragment 314 $[M\text{-}62]^-$. The loss of a third methoxy group produces mainly the fragment ion *m/z* 282 $[M\text{-}94]^-$. The loss of all 4 methoxy groups produces ion *m/z* 251 $[M\text{-}125]^-$. This is in complete agreement with previously published results (110) and the theoretical fragmentation pattern displayed in Figure 15. The different B[*a*]P-TME isomers, *trans/anti* $[^{2}H_8]$B[*a*]P-TME, and *trans/anti* $[^{13}C_3]_4$B[*a*]P-TME gave identical fragmentation patterns. GC-NICI-MS chromatograms and MS spectra are shown in Appendix I. In addition, about 7% of *trans/anti* B[*a*]P-TME fragments in the injector caused additional peaks at retention times 19.55, 19.61 and 21.68 min. These correspond to two isomers of the trimethoxy-B[*a*]P-TME and one dimethoxy B[*a*]P TME (Appendix I).

Table 7: HPLC Retention times of B[*a*]P TME isomers

	HPLC		**GCMS**	
	RT	**Melikian 1996[a]**	**RT**	**Melikian 1996[a]**
trans/anti B[*a*]P-TME	14.40	85.71	18.84	22.54
cis/anti B[*a*]P-TME	15.25	86.50	19.46	23.43
trans/syn B[*a*]P-TME	17.51	92.00	18.23	21.67
cis/syn B[*a*]P-TME	16.23	91.20	18.77	22.54

[a] The retention times [min] are compared to those reported by Melikian et al. (160).



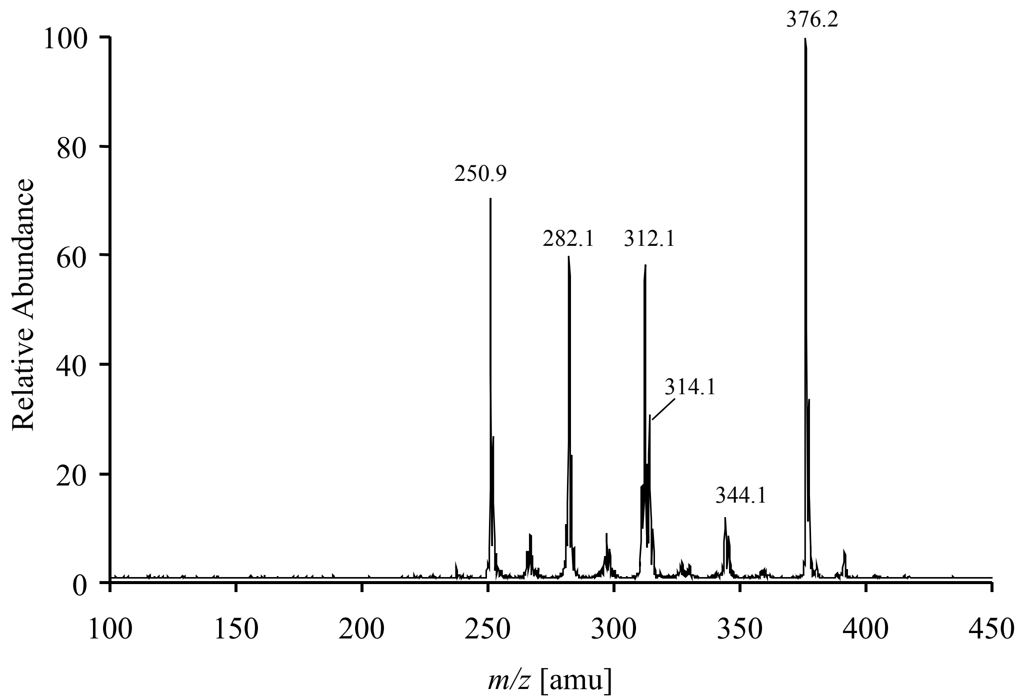


Figure 14: (**A**) GC-MS Chromatogram of *trans/anti* B[*a*]P-TME standard. (**B**) Mass spectrum of *trans/anti* B[*a*]P-TME standard peak at 18.84 min.

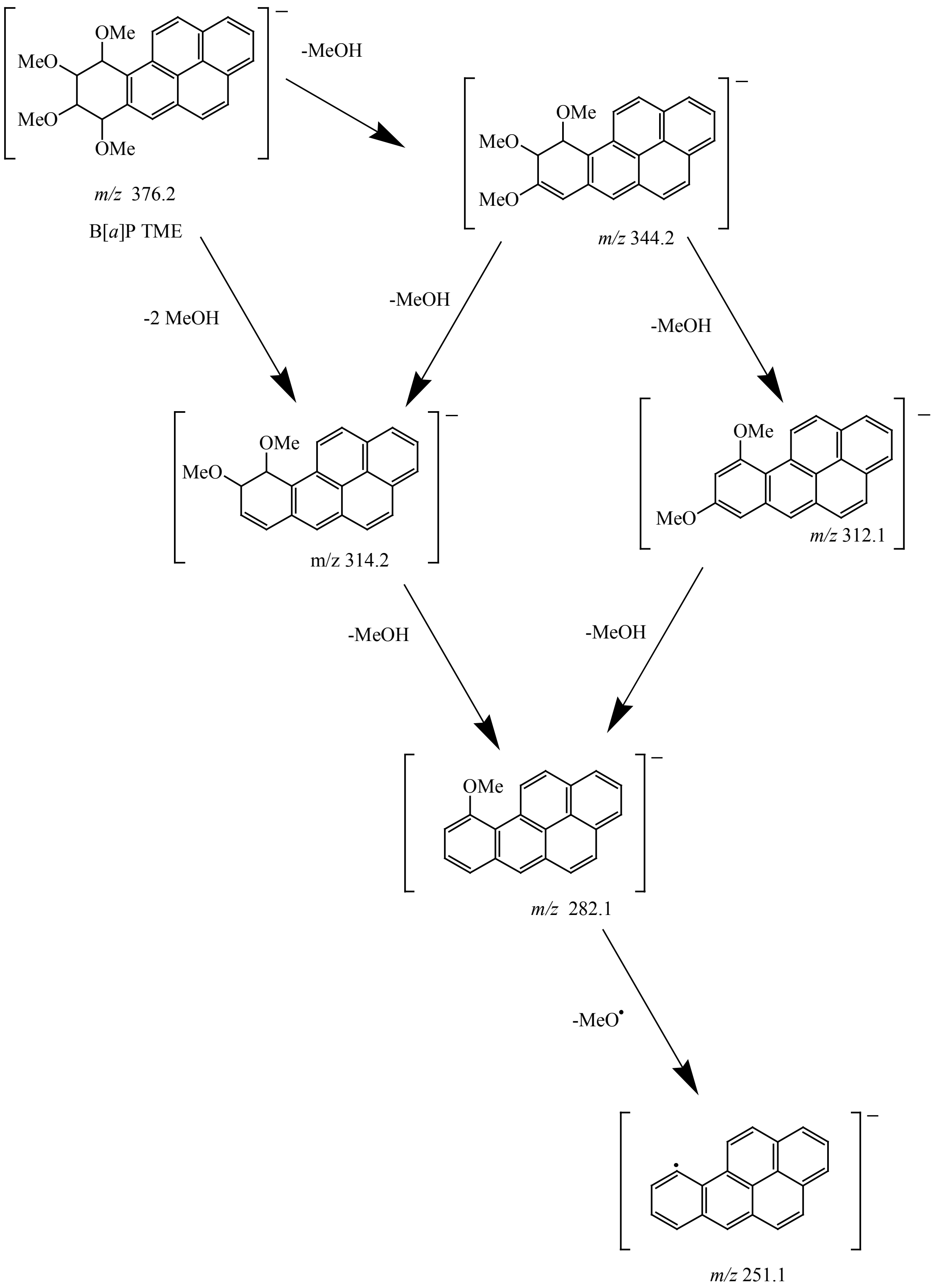


Figure 15: Fragmentation pattern of the B[*a*]P-TME derivatives.

3.2 Establishment of methods for the detection of BPDE-DNA adducts

3.2.1 BPDE-DNA adducts by HPLC-FD

A limit of detection (LOD, signal to noise ratio (S/N) greater than 2), of 2-5 fmol was repeatedly achieved with synthetic standards for all isomers. The recovery of the different B[*a*]P-tetraol isomers was >95% (data not shown). One mg *c.t.*DNA samples spiked with BPDE-DNA, representing 5, 25 and 100 fmol B[*a*]P-tetraol-releasing adducts, were analyzed to determine accuracy and precision. The *trans/anti* B[*a*]P-tetraols were released upon mild acid hydrolysis and quantified by HPLC-FD with an external standard curve prepared with synthetic B[*a*]P tetraols. The coefficient of variance (CV) for the detection of 5, 25 and 100 fmol were 18.2% 11.2% and 5 % respectively (Table 8). This clearly shows that the HPLC-FD method is capable of detecting 5 fmol in a 1 mg DNA sample corresponding to ~0.2 adducts/ 10^8nucleotides with a CV of < 20%.

Table 8: Accuracy of the analysis of B[*a*]P tetraols by HPLC-FD method

Sample	Peak area	B[*a*]P detected [fmol]	mean SD	% CV
5-1	1.318	3.77		
5-2	1.750	5.00		
5-3	1.344	3.84		
5-4	1.802	5.15		
5-5	1.996	5.70	4.69 ±0.85	18.2%
25-1	8.716	24.90		
25-2	8.792	25.12		
25-3	6.922	19.78		
25-4	7.953	22.72		
25-5	7.075	20.21	22.55 ±2.52	11.2%
100-1	42.950	122.71		
100-2	39.184	111.95		
100-3	37.502	107.15		
100-4	40.338	115.25		
100-5	39.731	113.52	114.12 ±5.67	5.0%

One mg *c.t.*DNA was spiked with BPDE-DNA representing 5, 25 or 100 fmol *trans/anti* B[*a*]P-tetraol-releasing adducts.

3.2.2 BPDE-DNA and protein adducts by GC-MS

A LOD (S/N >2) of 1 fmol/injection was repeatedly achieved. The recovery ranged from 10%-41%, leading to an overall LOD of 12-50 fmol/sample (adjusted for injection volume, 4 out of 10 µl). To study the accuracy of the GC-NICI-MS method we analyzed samples of 1 mg *c.t.*DNA spiked with BPDE-DNA, representing 5, 25 and 100 fmol B[*a*]P-tetraol releasing adducts. The released B[*a*]P-tetraols were derivatized to tetramethoxy ethers, as described in Materials and Methods (section 2.7), and quantified utilizing 1 pmol [$^{2}H_{8}$]B[*a*]P-tetraols as internal standard. B[*a*]P-TME was not detected in the 5 fmol samples, presumably due to the low recovery. The CVs for the detection of 25 and 100 fmol were 22.6% and 18%, respectively (Table 9). This clearly shows that the GC-MS method is capable of detecting 25 fmol in a 1 mg DNA sample correspond to ~0.8 adducts/ 10^{8}nucleotides, with a CV of < 25%. This method was then applied to analyze B[*a*]P-tetraol releasing DNA adducts in lungs of rats treated with NNK plus B[*a*]P (Figure 16).

Table 9: Accuracy of the analysis of B[*a*]P tetraols by GC-MS method

Sample	Area *m/z* 376 / Area *m/z* 384	B[*a*]P TME detected [fmol]	mean ± SD	% CV
5-1		n.d		
5-2		n.d		
5-3		n.d		
5-4		n.d		
5-5		n.d		
25-1	0.24	20.3		
25-2	0.24	20.2		
25-3	0.25	21.3		
25-4	1.01	86.8[a]		
25-5	0.36	31.0	23.2 ±5.24	22.6%
100-1	0.84	72.5		
100-2	1.40	120.4		
100-3	1.08	93.1		
100-4	1.19	102.4		
100-5	1.10	94.1	96.5 ±17.34	18.0%

One mg *c.t.*DNA was spiked with BPDE-.DNA, representing 5, 25 or 100 fmol *trans/anti* B[*a*]P-tetraol-releasing adducts.
[a] Data point was excluded with CI >95%, according to Q-test.

3.2.3 Analysis of liver DNA by HPLC-FD, GC-NICI-MS

To evaluate the suitably of the HPLC-FD and GC-NICI-MS methods for the analysis of BPDE-adducts *in vivo*, BPDE-DNA and 4 liver DNA samples from 4 mice treated with 3µmol B[*a*]P 24 h prior to sacrifice were analyzed. All three methods gave essentially the same results, as shown in Table 10. DNA reacted with BPDE was used as the control and additionally analyzed by LC-MS to verify that the B[*a*]P-tetraol released adducts represented the BPDE-N^2-dG adduct. The results clearly shows that both methods give essentially the same results for released B[*a*]P-tetraols. At this point the HPLC-FD method was handed over to a co-worker for the analysis of the mouse DNA samples and the GC-NICI-MS methods were applied to the rat samples (see below).

Table 10: Comparison of HPLC-FD, GC-NICI-MS and LC-MS for the detection of BPDE-DNA adduct in different samples

Sample	HPLC-FD[a]	GC-MS[b]	LC-MS[c]
BPDE treated c.t. DNA	1220 ± 9.2	1220 ±125	1388 ±654
Mouse liver DNA 1	0.85	0.95	NA
Mouse liver DNA 2	0.78	0.99	NA
Mouse liver DNA 3	0.99	0.97	NA
Mouse liver DNA 4	0.96	0.96	NA

Reported are the mean ± SD (n=5), except were noted
[a] Analysis of released B[*a*]P tetraols
[b] Analysis of released B[*a*]P tetraols derivatized to B[*a*]P-TMEs
[c] Analysis of B[*a*]PDE-3'-dGMP after enzyme hydrolysis (n=2)
NA= not analyzed because there was not sufficient DNA

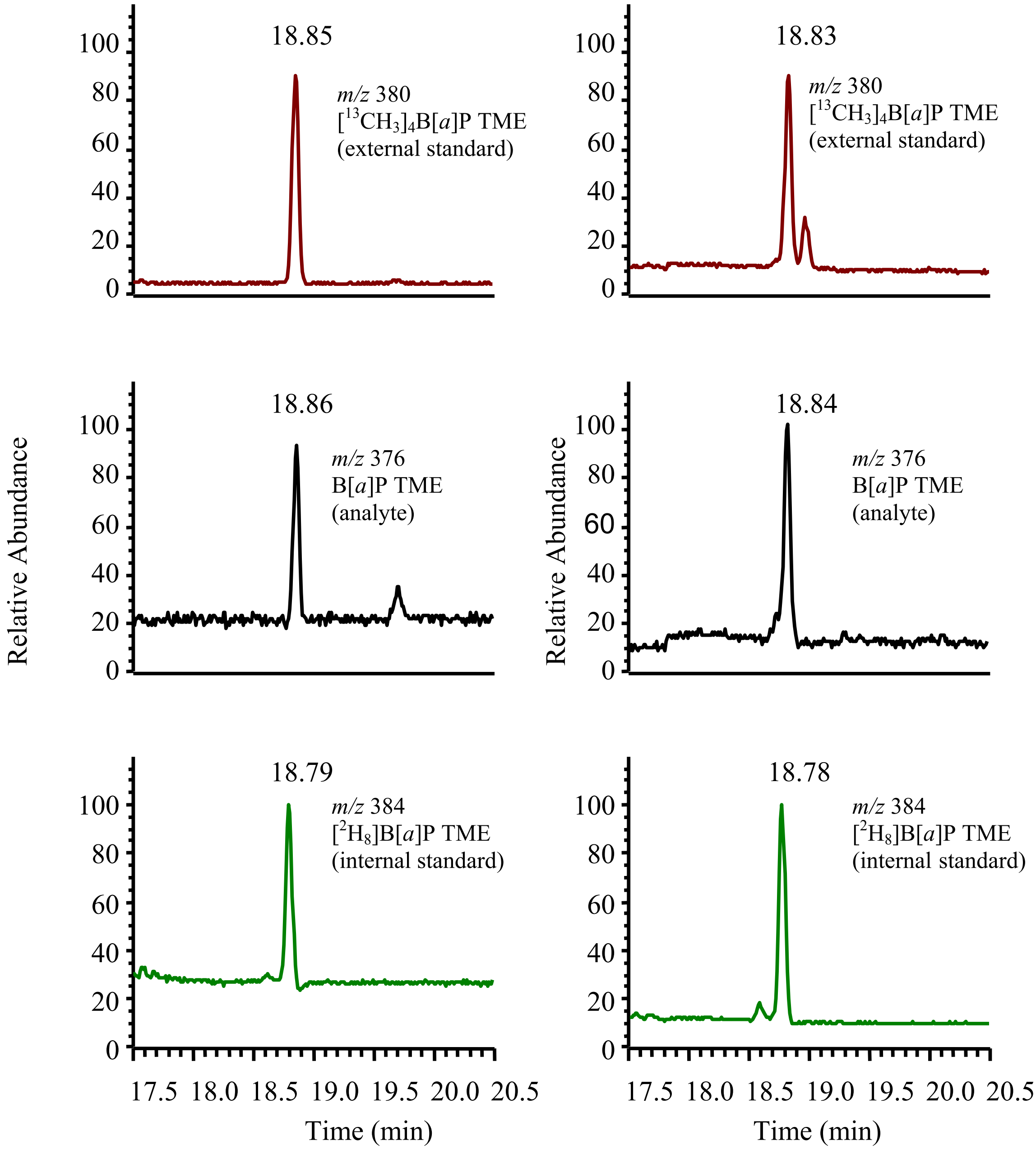


Figure 16: GC-NICI-MS analysis of B[*a*]P-TME derivative. Shown are the single ion chromatograms of a standard mixture (left panel) and a rat lung DNA sample (right panel).

3.3 HPB adduct assay validation

To validate the analysis for HPB–releasing adducts, tests were performed to check reproducibility, linearity and sensitivity. The reproducibility and linearity of the method were determined by analysis of HPB-released from hemoglobin (Hb) and DNA spiked with synthetic HPB.

3.3.1 Determination of precision

Precision was determined by analyzing samples of 1 mg *c.t.*DNA spiked with 100, 200, 250 or 300 fmol HPB respectively in quintuplets. The solutions were analyzed according to the procedure described in Materials and Methods (see section 2.9). CVs was <10% for all levels. Accuracy was within 10%.

Table 11: Accuracy of the analysis of HPB-releasing adducts by GC-MS

Sample	Area *m/z* 359 / Area *m/z* 361	HPB detected [fmol/sample]	mean ± SD	% CV
100-1	0.30	88.8		
100-2	0.32	95.6		
100-3	0.31	92.3		
100-4	0.32	96.7		
100-5	0.31	93.3	93.34 ±3.09	3.0%
200-1	0.72	214.5		
200-2	0.70	209.1		
200-3	0.79	237.8		
200-4	0.76	229.3		
200-5	0.65	194.5	217.04 ±17.02	8.0%
250-1	0.85	255.1		
250-2	0.82	246.8		
250-3	0.83	250.0		
250-4	0.86	259.2		
250-5	0.81	244.1	251.04 ±6.13	2.0%
300-1	0.96	288.7		
300-2	0.95	285.4		
300-3	1.04	313.4		
300-4	1.02	305.2		
300-5	0.90	270.2	292.58 ±17.03	6.0%

One mg *c.t.*DNA was spiked with HPB, representing 100, 200, 250 or 300 fmol.

3.3.2 Linearity of HPB-releasing DNA adducts

One mg of *c.t.*DNA was spiked with different amounts of HPB. All H_2O blanks contained a peak co-eluting with HPB-PFB. This peak represented of 3 ± 2 fmol/ sample (mean ± SD, n=3) and was subtracted from each amount detected. A strong linear correlation was achieved (R≅1.0) over the range of 3-100 fmol between the amount detected and amount added. The precision was <10% for all samples spiked with more than 5 fmol.

In later studies we found that the background varied with the location where the assays were carried out. Therefore, parallel to each set of samples (n=20), 3 water blanks were included. Theses blanks together gave a mean ± SD background of 20 ± 6 fmol HPB/sample analyzed in a laboratory dedicated for "human trace analysis" and 75 ± 15 fmol HPB/sample analyzed in the general laboratory. Strong efforts in reducing or eliminating this background were unsuccessful (data not shown).

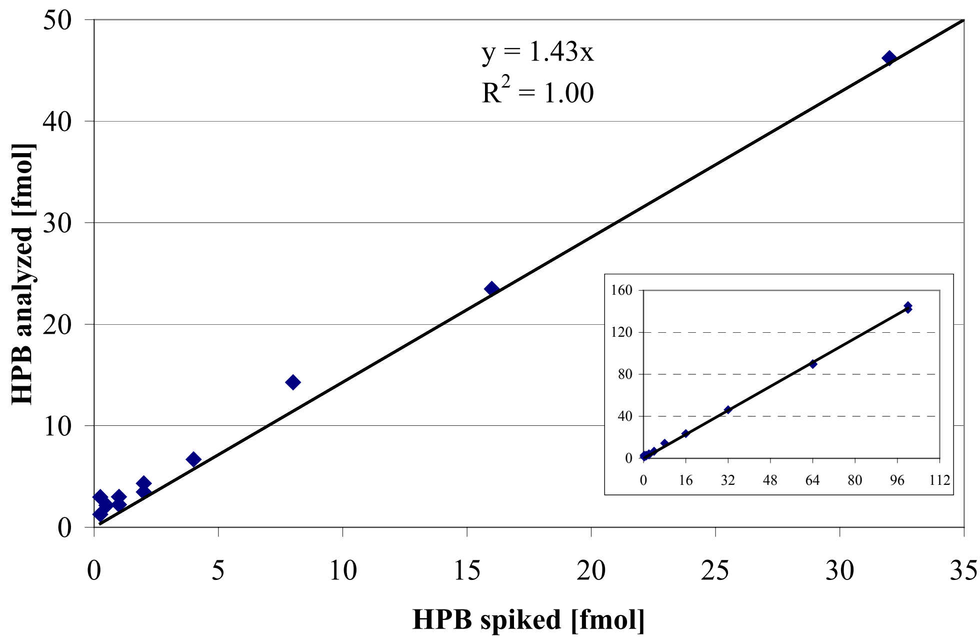


Figure 17: Linearity of HPB detected in aliquots from 100 µg calf thymus DNA solution spiked with various amounts of synthetic HPB. The insert shows the extended graph up to 100 fmol HPB spiked.

3.3.3 HPB releasing Hb adducts

One gram of human Hb obtained from a non-smoking volunteer was spiked with different amounts of synthetic HPB. All water blanks contained a background peak co-eluting with HPB-PFB. This background corresponded to 25 ± 3.1 fmol/sample. The mean (n=3) of the background was subtracted from each amount detected. A linear correlation (R≅0.97) between the amount detected and amount added was achieved. The CV was <20% overall and <10% for values above 100 fmol, presumably due to less influence of the background at higher levels.

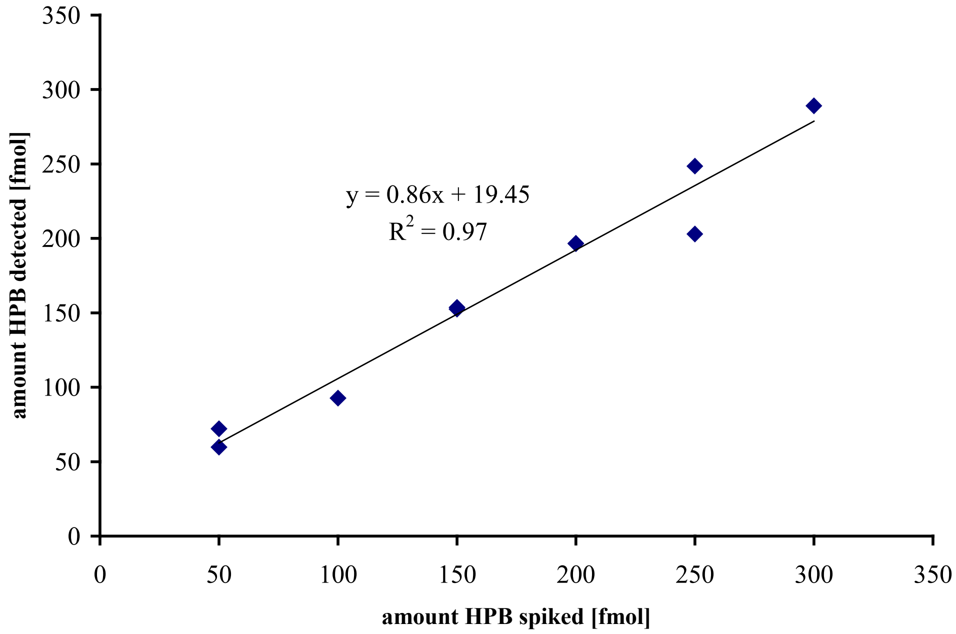


Figure 18: Linearity of HPB detected in aliquots of hemoglobin solutions spiked with various amounts of HPB.

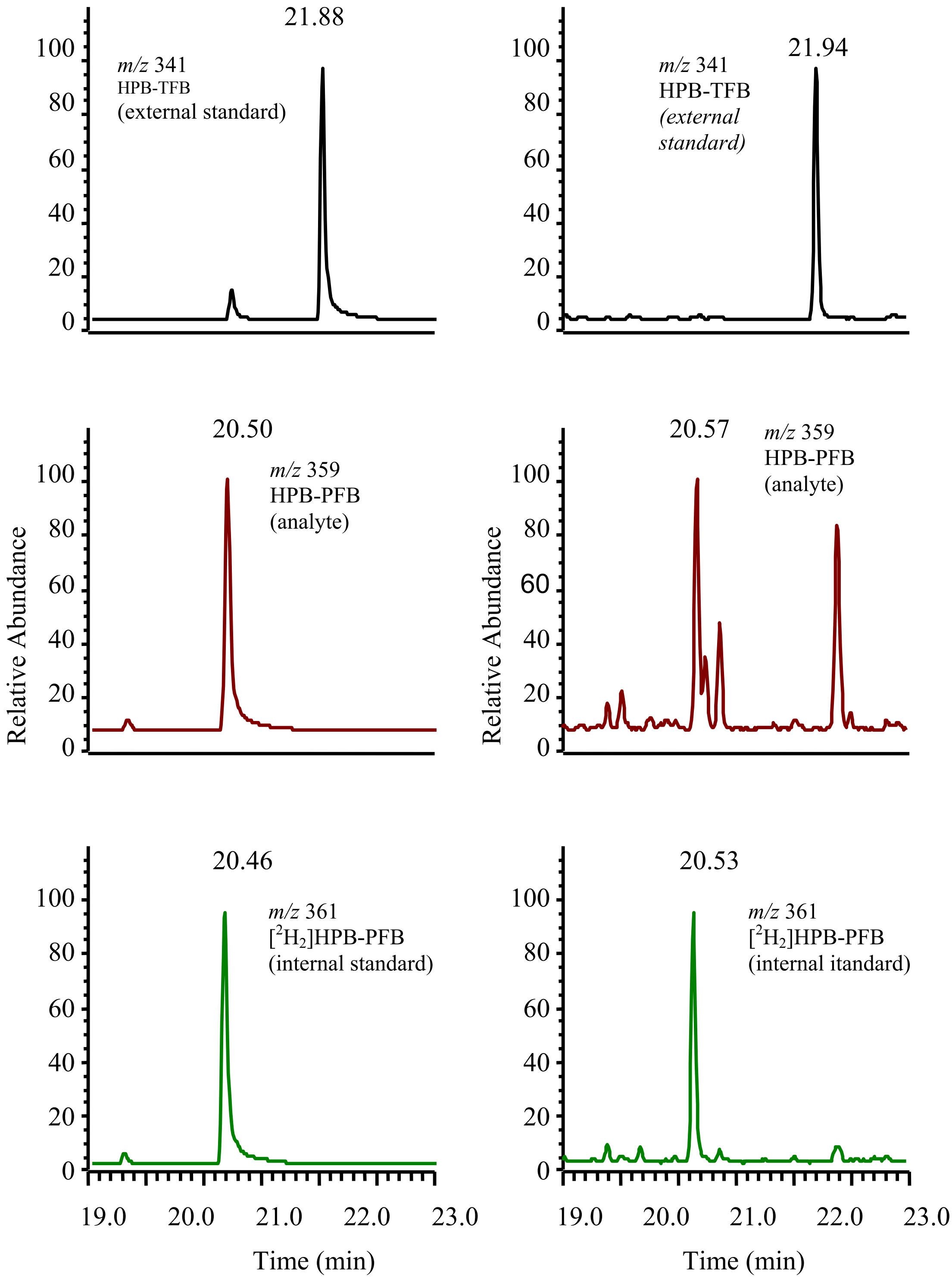


Figure 19: GC-NICI-MS analysis of HPB-PFB derivatives. Shown are selected ion chromatograms of a standard mixture (left panel) and rat lung DNA sample (right panel).

3.4 DNA adduct formation in lungs of A/J mice treated with NNK and B[*a*]P alone or in combination

In the first animal study, the goal was to determine whether or not adduct formation is different when NNK and B[*a*]P are given individually or in combination. α-Hydroxylation of NNK at the methyl carbon produces pyridyloxobutyl DNA adducts, which can be hydrolyzed to release HPB (42). The released HPB is then derivatized with PFBC and quantified by GC-NICI-MS. α-Hydroxylation of NNK at the methylene carbon leads to methyl DNA adducts. Among them, O^6-mG is thought to be important in tumor induction in mice (43). B[*a*]P produces mainly the BPDE-N^2-dG adduct, which releases predominantly *trans/anti* B[*a*]P-tetraol upon acid hydrolysis (88). O^6-mG and released B[*a*]P-tetraols were quantified by HPLC-FD[1].

Mice were treated either with cottonseed oil only, B[*a*]P, NNK or NNK plus B[*a*]P in cottonseed oil by gavage once a week for 1, 4 or 8 weeks and sacrificed 24 h after the last carcinogen administration (Figure 20, Table 12). HPB-releasing adduct levels increased steadily over time, however, due to the small number of animals per group, this trend was not significant. Adduct levels were similar in the groups receiving NNK or NNK plus B[*a*]P. Adducts were not detected in the control animals. One animal in the group receiving a single dose of B[*a*]P had a small amount of HPB-releasing DNA adduct, presumably due to the assay background. O^6-mG adduct levels increased over time and adduct levels were similar when NNK was give alone or in combination with B[*a*]P. The B[*a*]P-tetraol-releasing adduct levels were increased between 1 and 4 weeks of treatment, but remained steady after that. There was no significant effect of NNK on BPDE-N^2-dG adduct levels or *vice versa*. Collectively, these results demonstrate that DNA adduct formation by a mixture of B[*a*]P and NNK was similar to that observed when the carcinogens were given alone.

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[1] The analysis of the O^6-mG and the B[a]P-tetraol-releasing DNA adduct was completed by co-workers: Sticha, K. Hong, L. and Su, X.

Table 12: DNA adducts in lung of A/J mouse lung 24 h after 1, 4 or 8 weekly gavage treatments with NNK and B[*a*]P alone or in combination.

group	DNA adduct levels [fmol/µg DNA] [a]		
	HPB-releasing	*O*^{6}-mG	B[*a*]P-tetraol-releasing
1 week (single dose)			
None	ND[b]	ND.	ND
NNK	0.63 ±0.06	2.63 ±0.05	ND
B[*a*]P	0.22[c]	ND	0.48 ±0.14
NNK plus B[*a*]P	0.60 ±0.41	2.08 ±0.68	0.36 ±0.20
4 weeks			
None	ND	ND	ND
NNK	1.91 ±1.12	6.70 ±1.23	
B[*a*]P	ND	ND	1.55 ±0.40
NNK plus B[*a*]P	1.32 ±0.39	5.21 ±0.91	1.33 ±0.12
8 weeks			
None	ND	ND	ND
NNK	3.41 ±2.44	8.87 ±1.10	ND
B[*a*]P	ND	ND	1.31 ±0.53
NNK plus B[*a*]P	3.33 ±1.71	7.64 ±1.72	2.30 ±1.16

NNK, B[*a*]P or NNK plus B[*a*]P were given weekly by gavage (3 µmol each in 0.2 ml cotton seed oil) for 1, 4 or 8 weeks. Reported are the mean ± SD (n=3)

[a] The analysis of the *O*^{6}-MG and the B[*a*]P-tetraol-releasing DNA adduct were completed by co-workers

[b] ND., not detected

[c] One animal had a detectable amount HPB of 0.67 fmol/µg DNA.

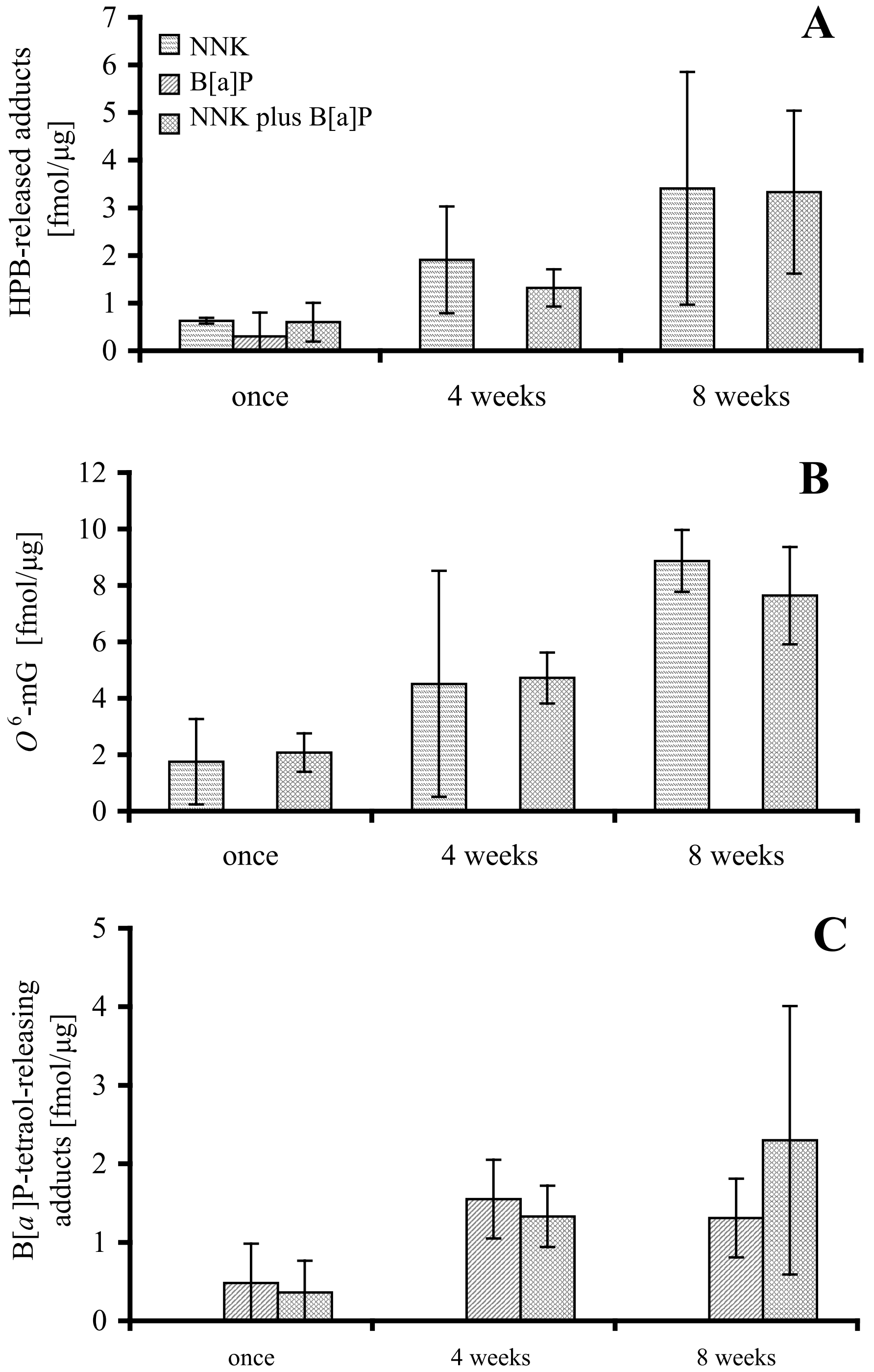

Figure 20: DNA adducts in mouse lung after gavage treatments of NNK and B[*a*]P (3 µmol each) alone or in combination. The analysis of the O^6-mG and B[*a*]P-tetraol-releasing DNA adduct were completed by co-workers. Each bar represents the mean ± SD (n=3). [a] One sample had detectable amount of HPB (0.67 fmol/µg DNA).

3.5 Effects of PEITC and BITC on DNA adduct formation from NNK plus B[*a*]P in lung of A/J mice

In the second animal study, the effects of PEITC and BITC, given by gavage or in the diet, on adduct formation, were evaluated.

3.5.1 Effects of gavaged PEITC plus BITC

A/J mice were given PEITC and/or BITC 2 h prior to carcinogen administration once weekly for 1, 4 or 8 weeks, and subgroups were sacrificed 4, 24 or 120 h after the last carcinogen treatment. A mean ± SD of 91.4 ± 46.0 µg DNA per sample was analyzed for HPB-releasing adducts. In the analysis of HPB-releasing adducts, the mean background was 68.8 ± 12.0 fmol/sample (n=6), and was subtracted from each detected amount. The adduct levels were expressed as fmol HPB released (corrected for background)/µg DNA. The average recovery of 4,4-[$^2H_2$]HPB was 27.5 ± 17.3%. The mean level detected was 188.2 ±152. fmol/ sample and was 3-fold above the background. The effects of ITCs on HPB-releasing adduct levels were weak and not always significant. A non-significant inhibitory effect on the formation of adducts was observed in some groups treated for 4 or 8 weeks. In these groups, the reduction of HPB-releasing adduct levels was strongest in the mice receiving PEITC alone. Increased levels of BITC may have counteracted this effect, however due to large variations within the groups, most differences were not significant (Figure 21, Table 13).

There were no inhibitory effects of gavaged ITCs on levels of O^6-mG. A trend towards increased O^6-mG adduct levels was observed and this was significant in two instances (Figure 22, Table 13).

B[*a*]P-tetraol-releasing adduct levels were significantly decreased by all combinations of PEITC plus BITC, 120 h after the last carcinogen treatment. At the 24 h interval, B[*a*]P-tetraol-releasing adduct levels were decreased, but this was not always significant (Figure 23, Table 13). There was no effect on the adduct levels 4 h after the last carcinogen treatment.

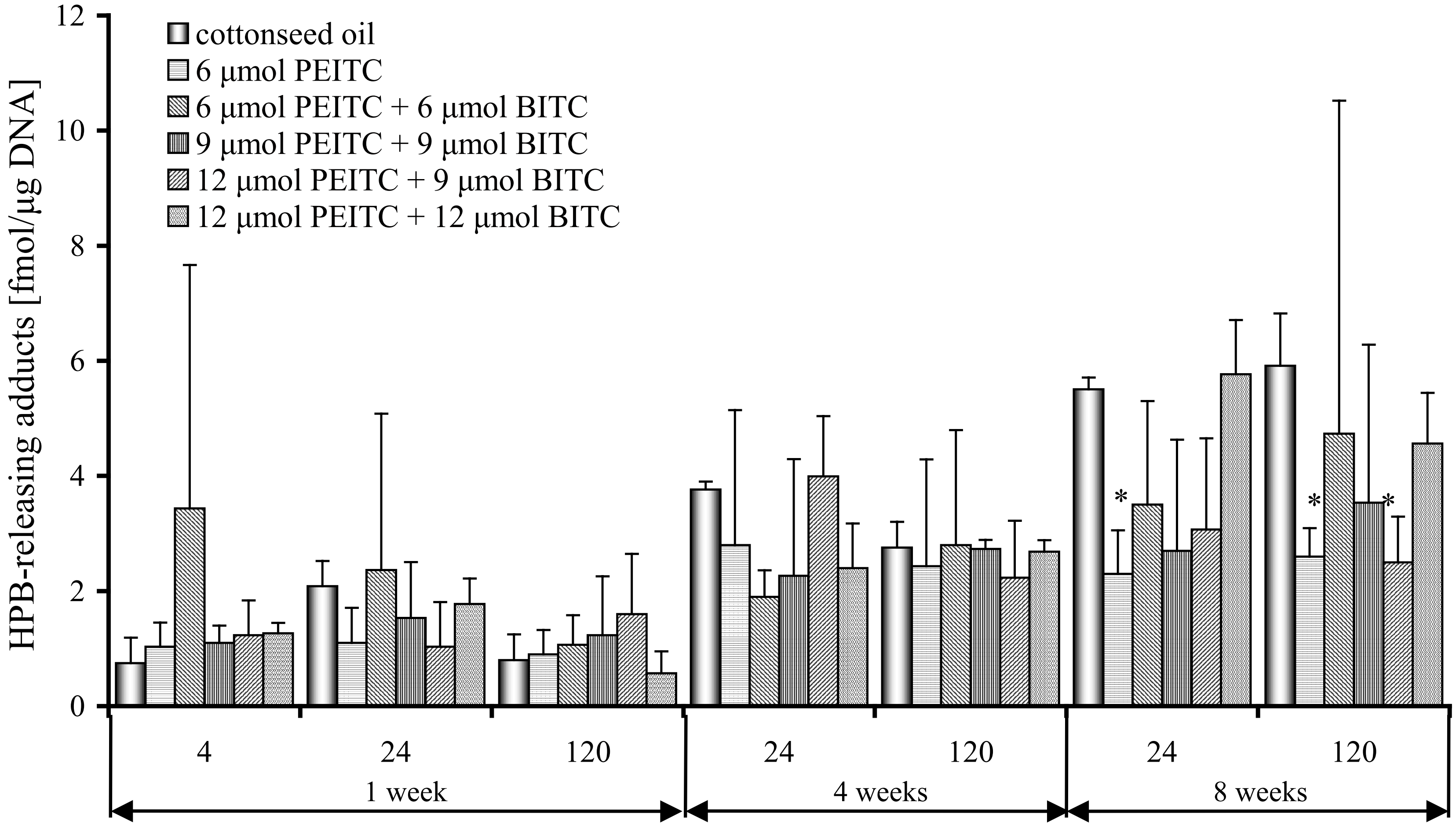

Figure 21: HPB-releasing DNA adduct levels in A/J mice lung after 1, 4 or 8 weekly gavage treatments of NNK plus B[*a*]P (3 µmol each) and mixtures of PEITC and BITC (doses as indicated) 2 h prior to the carcinogens. Each bar represents the mean ± SD (n=3). *,$P<0.05$.

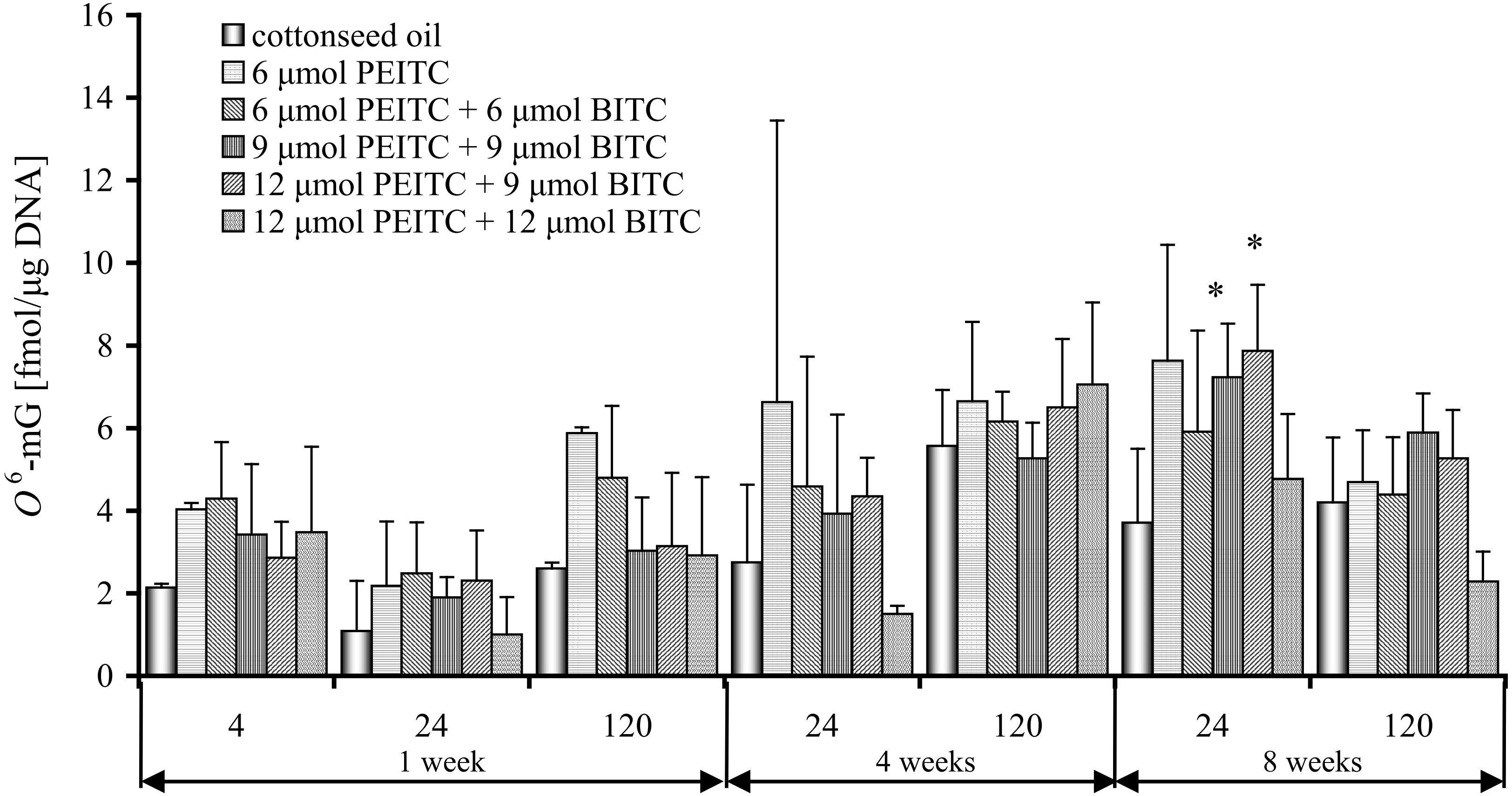

Figure 22: O^6-mG adduct levels in A/J mice lung after 1, 4 or 8 weekly gavage treatments of NNK plus B[*a*]P (3 µmol each) and mixtures of PEITC and BITC (doses as indicated) 2 h prior to the carcinogens. Each bar represents the mean ± SD (n=3). This analysis was completed by co-workers.

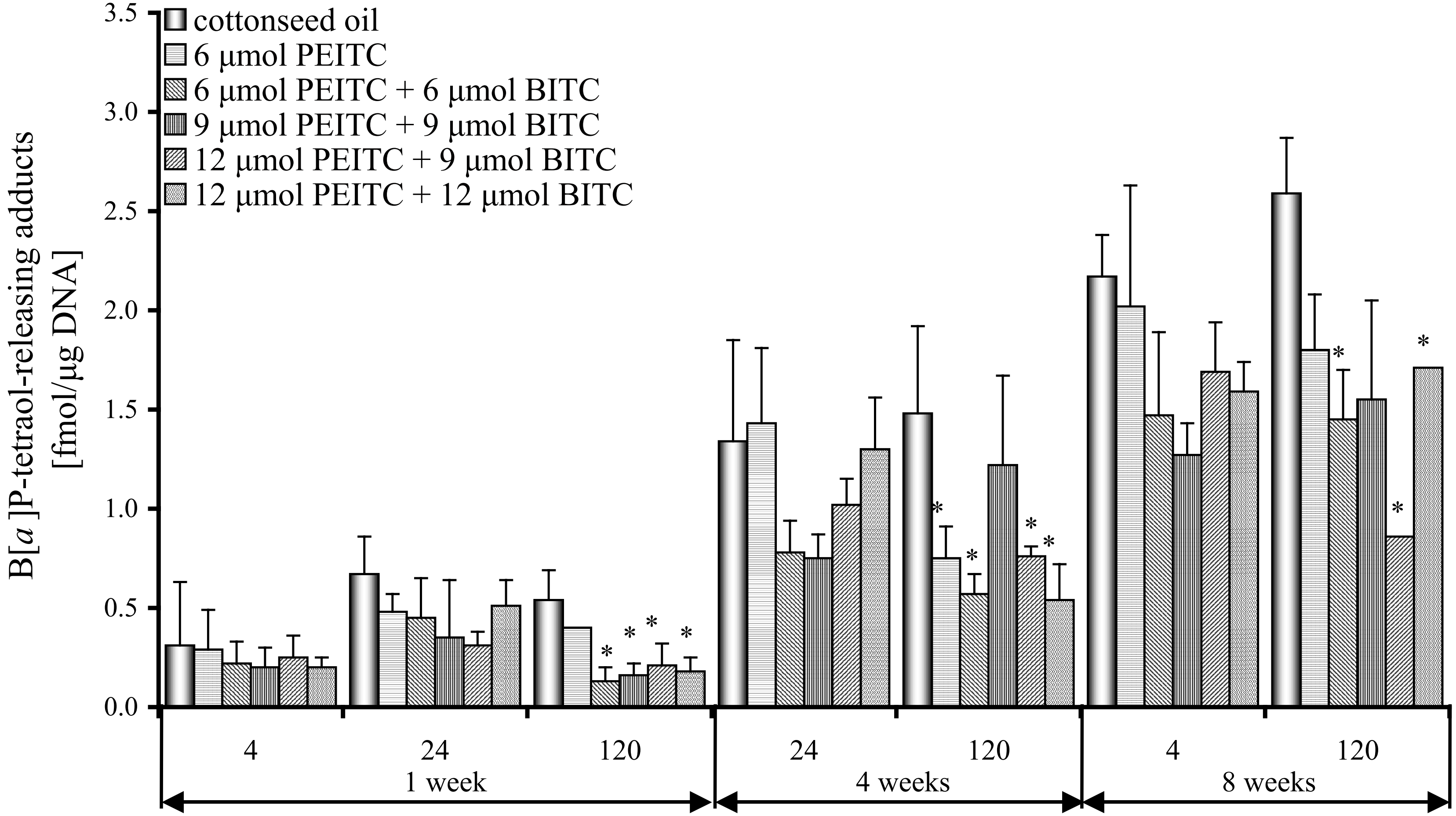

Figure 23: B[*a*]P-tetraol releasing DNA adduct levels in A/J mouse lung after 1, 4 or 8 weekly gavage treatments of NNK plus B[*a*]P (3 µmol each) and mixtures of PEITC and BITC (doses as indicated) 2 h prior to the carcinogens. Each bars represents the mean ± SD (n=3). This analysis was completed by co-workers.

Table 13: DNA adduct levels in lung of A/J mouse treated with NNK plus B[*a*]P and PEITC plus BITC by gavage

Duration Sacrifice[a]	Isothiocyanates treatment	adduct level [fmol/µg][b] HPB	O^6-mG	B[*a*]P-tetraol
Once				
4	cotton seed oil	0.74 ± 0.45	2.14 ± 0.09	0.31 ± 0.32
4	6 µmol PEITC	1.03 ± 0.42	4.03 ± 0.16	0.29 ± 0.20
4	6 µmol PEITC + 6 µmol BITC	3.43 ± 4.23	4.29 ± 1.37	0.22 ± 0.11
4	9 µmol PEITC + 9 µmol BITC	1.10 ± 0.30	3.42 ± 1.71	0.2 ± 0.10
4	12 µmol PEITC + 9 µmol BITC	1.23 ± 0.60	2.86 ± 0.87	0.25 ± 0.11
4	12 µmol PEITC + 12 µmol BITC	1.27 ± 0.18	3.48 ± 2.07	0.20 ± 0.05
24	cotton seed oil	2.09 ± 0.44	1.09 ± 1.21	0.67 ± 0.19
24	6 µmol PEITC	1.10 ± 0.61	2.18 ± 1.56	0.48 ± 0.09
24	6 µmol PEITC + 6 µmol BITC	2.37 ± 2.71	2.48 ± 1.24	0.45 ± 0.20
24	9 µmol PEITC + 9 µmol BITC	1.53 ± 0.97	1.90 ± 0.49	0.35 ± 0.29
24	12 µmol PEITC + 9 µmol BITC	1.03 ± 0.78	2.31 ± 1.21	0.31 ± 0.07
24	12 µmol PEITC + 12 µmol BITC	1.77 ± 0.44	1.00 ± 0.91	0.51 ± 0.13
120	cotton seed oil	0.80 ± 0.45	2.60 ± 0.14	0.54 ± 0.15
120	6 µmol PEITC	0.90 ± 0.42	5.88 ± 0.14	0.40 [c]
120	6 µmol PEITC + 6 µmol BITC	1.07 ± 0.51	4.80 ± 1.74	0.13 ± 0.07*
120	9 µmol PEITC + 9 µmol BITC	1.23 ± 1.02	3.03 ± 1.29	0.16 ± 0.06*
120	12 µmol PEITC + 9 µmol BITC	1.60 ± 1.04	3.14 ± 1.78	0.21 ± 0.11*
120	12 µmol PEITC + 12 µmol BITC	0.57 ± 0.38	2.92 ± 1.89	0.18 ± 0.07*
4 weeks				
24	cotton seed oil	3.76 ± 0.14	2.75 ± 1.88	1.34 ± 0.51
24	6 µmol PEITC	2.80 ± 2.34	6.63 ± 6.82	1.43 ± 0.38
24	6 µmol PEITC + 6 µmol BITC	1.90 ± 0.46	4.59 ± 3.14	0.78 ± 0.16
24	9 µmol PEITC + 9 µmol BITC	2.27 ± 2.02	3.93 ± 2.40	0.75 ± 0.12
24	12 µmol PEITC + 9 µmol BITC	3.99 ± 1.04	4.35 ± 0.93	1.02 ± 0.13
24	12 µmol PEITC + 12 µmol BITC	2.40 ± 0.77	1.50 ± 0.20	1.30 ± 0.26
120	cotton seed oil	2.75 ± 0.45	2.75 ± 1.88	1.48 ± 0.44
120	6 µmol PEITC	2.43 ± 1.86	5.57 ± 1.35	0.75 ± 0.16*
120	6 µmol PEITC + 6 µmol BITC	2.80 ± 2.00	6.65 ± 1.92	0.57 ± 0.10*
120	9 µmol PEITC + 9 µmol BITC	2.73 ± 0.15	6.16 ± 0.72	1.22 ± 0.45
120	12 µmol PEITC + 9 µmol BITC	2.23 ± 0.99	5.27 ± 0.86	0.76 ± 0.05*
120	12 µmol PEITC + 12 µmol BITC	2.68 ± 0.20	6.50 ± 1.66	0.54 ± 0.18*
8 weeks				
24	cotton seed oil	5.50 ± 0.21	3.71 ± 1.79	2.17 ± 0.21
24	6 µmol PEITC	2.30 ± 0.75*	7.63 ± 2.81	2.02 ± 0.61
24	6 µmol PEITC + 6 µmol BITC	3.50 ± 1.80	5.91 ± 2.45	1.47 ± 0.42
24	9 µmol PEITC + 9 µmol BITC	2.70 ± 1.93	7.23 ± 1.30*	1.27 ± 0.16
24	12 µmol PEITC + 9 µmol BITC	3.07 ± 1.59	7.87 ± 1.60*	1.69 ± 0.25
24	12 µmol PEITC + 12 µmol BITC	5.77 ± 0.95	4.77 ± 1.57	1.59 ± 0.15
120	cotton seed oil	5.92 ± 0.91	4.20 ± 1.57	2.59 ± 0.28
120	6 µmol PEITC	2.60 ± 0.49*	4.69 ± 1.26	1.80 ± 0.28
120	6 µmol PEITC + 6 µmol BITC	4.73 ± 5.79	4.39 ± 1.39	1.45 ± 0.25*
120	9 µmol PEITC + 9 µmol BITC	3.53 ± 2.75	5.89 ± 0.95	1.55 ± 0.50
120	12 µmol PEITC + 9 µmol BITC	2.50 ± 0.79*	5.27 ± 1.17	0.86 [c*]
120	12 µmol PEITC + 12 µmol BITC	4.56 ± 0.88	2.29 ± 0.72	1.71 [c*]

3.5.2 Effects of dietary PEITC plus BITC

In the second part of animal study #2, PEITC and BITC were given in the diet starting one week prior to the carcinogen administrations and continuing until sacrifice (Figure 24, Table 14). PEITC and PEITC plus BITC had no effect on levels of HPB-releasing adducts at 4, 24 or 120 h intervals after a single carcinogen treatment. The adduct levels were reduced after 4 or 8 weekly treatments at both 24 and 120 h. These differences were not always significant, presumably due large variation within the groups and a small number of animals within the group (n=3). Similar to the gavage experiment, the levels of O^6-mG adduct levels were slightly increased after 1 or 4 weekly treatments; however, adduct levels were slightly decreased at 8 weeks, but none of these effects were significant. Dietary PEITC or PEITC plus BITC had little effect on B[*a*]P-tetraol-releasing adduct levels.

⇐ Legend to Table 13: NNK plus B[*a*]P doses were given weekly by gavage (3 μmol each in 0.2 ml cottonseed oil) for 1,4 or 8 weeks. Reported are the mean ± SD (n=3) except were noted. PEITC and BITC were given as indicated in 0.2 ml cottonseed oil) 2 h prior to the carcinogens by gavage.

[a] Represents time after the last carcinogen administration.

[b] The analysis of the O^6-mG and the B[*a*]P-tetraol-releasing DNA adduct was completed co-workers.

[c] reported are the mean (n=2)

[*] significantly different from control (p<0.05)

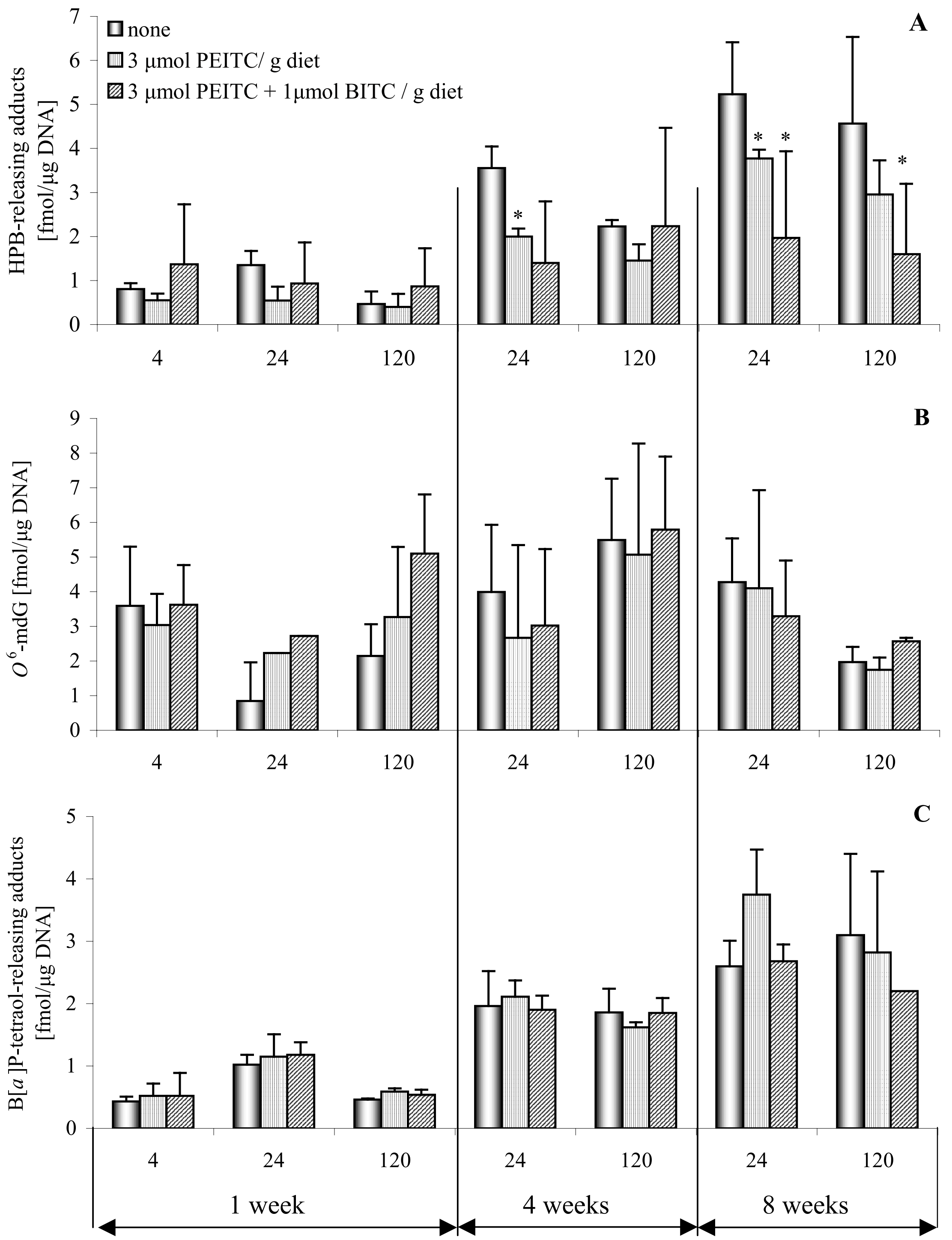

Figure 24: DNA adduct levels in lung of A/J mice treated with NNK plus B[*a*]P after 1, 4 or 8 weekly gavage treatments of. NNK plus B[a]P (3 µmol each) with or without administration of dietary PEITC (3µmol/g diet) and BITC (1 µmol/g diet) or PEITC plus BITC. Shown are HPB-releasing (A), O^6-mG (B), and B[*a*]P-tetraol-releaseing (C) adduct levels. Each bars represents the mean + SD (n=3). The analysis of O^6-mG and B[*a*]P tetraol releasing adduct were completed by co-workers.

Table 14: DNA adducts levels in lung of A/J mice treated with NNK plus B[*a*]P by gavage and PEITC plus BITC in the diet.

Duration	Sacrifice[a] [h]	Isothiocyanate treatment	Adduct level [fmol/µg][b] HPB	O^6-mG	B[*a*]P tetraol
1 week	4	None	0.81 ± 0.13	3.59 ± 1.71	0.43 ± 0.08
	4	3 µmol PEITC	0.55 ± 0.15	3.04 ± 0.90	0.52 ± 0.20
	4	3 µmol PEITC + 1 µmol BITC	1.37 ± 0.68	3.62 ± 1.15	0.52 ± 0.37
	24	None	1.35 ± 0.32	0.85 ± 1.11	1.02 ± 0.16
	24	3 µmol PEITC	0.55 ± 0.32	2.23	1.15 ± 0.36
	24	3 µmol PEITC + 1 µmol BITC	0.93 ± 0.42	2.72	1.18 ± 0.20
	120	None	0.47 ± 0.28	2.15 ± 0.91	0.46 ± 0.02
	120	3 µmol PEITC	0.40 ± 0.30	3.27 ± 2.02	0.59 ± 0.05
	120	3 µmol PEITC + 1 µmol BITC	0.87 ± 0.32	5.10 ± 1.71	0.54 ± 0.08
4 weeks	24	None	3.56 ± 0.49	3.99 ± 1.94	1.96 ± 0.56
	24	3 µmol PEITC	2.00 ± 0.18	2.67 ± 2.68	2.11 ± 0.26
	24	3 µmol PEITC + 1 µmol BITC	1.40 ± 0.52	3.02 ± 2.21	1.9 ± 0.23
	120	None	2.23 ± 0.15	5.49 ± 1.77	1.86 ± 0.38
	120	3 µmol PEITC	1.45 ± 0.37	5.07 ± 3.21	1.62 ± 0.08
	120	3 µmol PEITC + 1 µmol BITC	2.23 ± 1.17	5.79 ± 2.11	1.85 ± 0.24
8 weeks	24	None	5.23 ± 1.18	4.28 ± 1.26	2.60 ± 0.41
	24	3 µmol PEITC	3.77 ± 0.20	4.10 ± 2.83	3.75 ± 0.72
	24	3 µmol PEITC + 1 µmol BITC	1.97 ± 1.33	3.29 ± 1.61	2.68 ± 0.27
	120	None	4.57 ± 1.97	1.97 ± 0.44	3.10 ± 1.30
	120	3 µmol PEITC	2.96 ± 0.78	1.75 ± 0.35	2.82 [c]
	120	3 µmol PEITC + 1 µmol BITC	1.60 ± 0.62	2.57 ± 0.10	2.20 [c]

NNK plus B[*a*]P were given weekly by gavage (3 µmol each in 2.0 ml cottonseed oil) for 1, 4 or 8 weeks. PEITC and BITC were given in the diet (levels as indicated per g diet) starting one week prior to the carcinogen (at does indicated per g diet). Reported are the mean ± SD (n=3), except were noted.

[a] Indicates time after last carcinogen treatment.

[b] The analysis of the O^6-mG and the B[a]P-tetraol-releasing DNA adducts was completed by co-workers.

[c] reported are the mean (n=2).

3.6 Effects of PEITC and BITC on adduct formation from NNK plus B[*a*]P in rats

Our goal was to determine the effects of PEITC and/or BITC on the formation of HPB- and *trans/anti* B[*a*]P-tetraol-releasing DNA and protein adducts in rats treated with NNK plus B[*a*]P. This was accomplished using GC-NICI-MS techniques.

HPB releasing adducts

A mean ± SD amount of 97.1 ± 32.5 µg DNA was analyzed for HPB-releasing adducts. The mean ± SD background of HPB in the negative controls was 23.6 ± 6.6 fmol/sample (n=10), and was subtracted from each amount detected. The mean ± SD recovery of 4,4-[$^2H_2$]HPB was 20.1 ± 13.6%. The mean ± SD adduct level detected in lung and liver was 118.1 ± 60.7 and 71.2 ± 18.4 fmol/ sample, respectively, and was 5- and 3-fold above the background. The adduct levels were expressed as fmol HPB released (corrected for background)/µg DNA.

Table 15 summarizes the adduct levels for each group after 8 and 16 weeks of treatment. PEITC reduced the mean HPB-releasing DNA adduct levels in the lung after 8 and 16 weeks of treatment by 50%. The means ± SD were 1.6 ± 0.3, 1.3 ± 0.4, 0.6 ± 0.1 and 0.5 ± 0.4 for the groups treated with NNK plus B[*a*]P alone or in combination with BITC, PEITC, or PEITC plus BITC, respectively. After 16 weeks, similar results were obtained, confirming a reduction of HPB-releasing DNA adducts in the lung by ~50% in the groups treated with PEITC and PEITC plus BITC. The mean ± SD HPB-releasing DNA adduct levels in the liver specimens were not different among the groups, and ranged from 0.33 ± 0.12 to 0.56 ± 0.13 fmol/µg DNA. PEITC and PEITC plus BITC treatment reduced the levels of HPB-releasing DNA adducts in lung to levels similar to those seen in the liver DNA (Figure 25).

HPB-releasing hemoglobin adduct levels were 40-50% lower in the groups treated with PEITC or PEITC plus BITC compared to controls (Figure 26, Table 15). The HPB-releasing Hb adduct levels after 8 weeks of treatment were 56.1 ± 2.5, 55.8 ± 2.3, 24.4 ± 2.0, and 31.7 ± 5.8 in groups treated with NNK plus B[*a*]P alone or in combination with, BITC, PEITC and PEITC plus BITC, respectively. The 50% inhibition of HPB-releasing-Hb adducts is similar to that inhibition observed for HPB-releasing DNA adducts in lung. The inhibition of hemoglobin adducts was persistent until 12 weeks (Figure 26). After that, HPB-releasing adduct levels declined non-significantly in the NNK plus B[*a*]P and NNK plus B[*a*]P/BITC treated groups to the levels of the PEITC and PEITC plus BITC group. After 16 weeks of treatment, there were no differences in HPB-releasing hemoglobin adduct levels among the treatment groups.

*Trans/anti B[*a*]P-tetraol-releasing adducts*

A mean ± SD amount of 97.1 ± 32.5 μg DNA was analyzed for B[*a*]P-tetraol-releasing adducts. The recovery of the *trans/anti* [$^2H_8$]B[*a*]P-tetraol was 28.5± 15.2%. None of the H_2O blanks contained any detectable *trans/anti* B[*a*]P-TME and the spiked positive controls were within CV<10% (n=8, data not shown). There was no effect of PEITC and PEITC plus BITC on the formation of B[*a*]P-tetraol-releasing adducts in lung or liver (Figure 25). The adduct levels were higher in liver than in lung DNA, with a mean ± SD of 0.7 ± 0.4 and 1.1 ± 0.6 for lung and liver DNA, respectively (Table 15).

Trans/anti B[*a*]P-tetraol releasing globin adducts were also not affected by the ITC-treatment (Figure 26, Table 15). The variation within the groups was ~50% and was 2-fold greater than the CV of the assay (section 3.2.2, page 49). The B[*a*]P-tetraol-releasing globin adduct levels were similar to the HPB-releasing-Hb adducts.

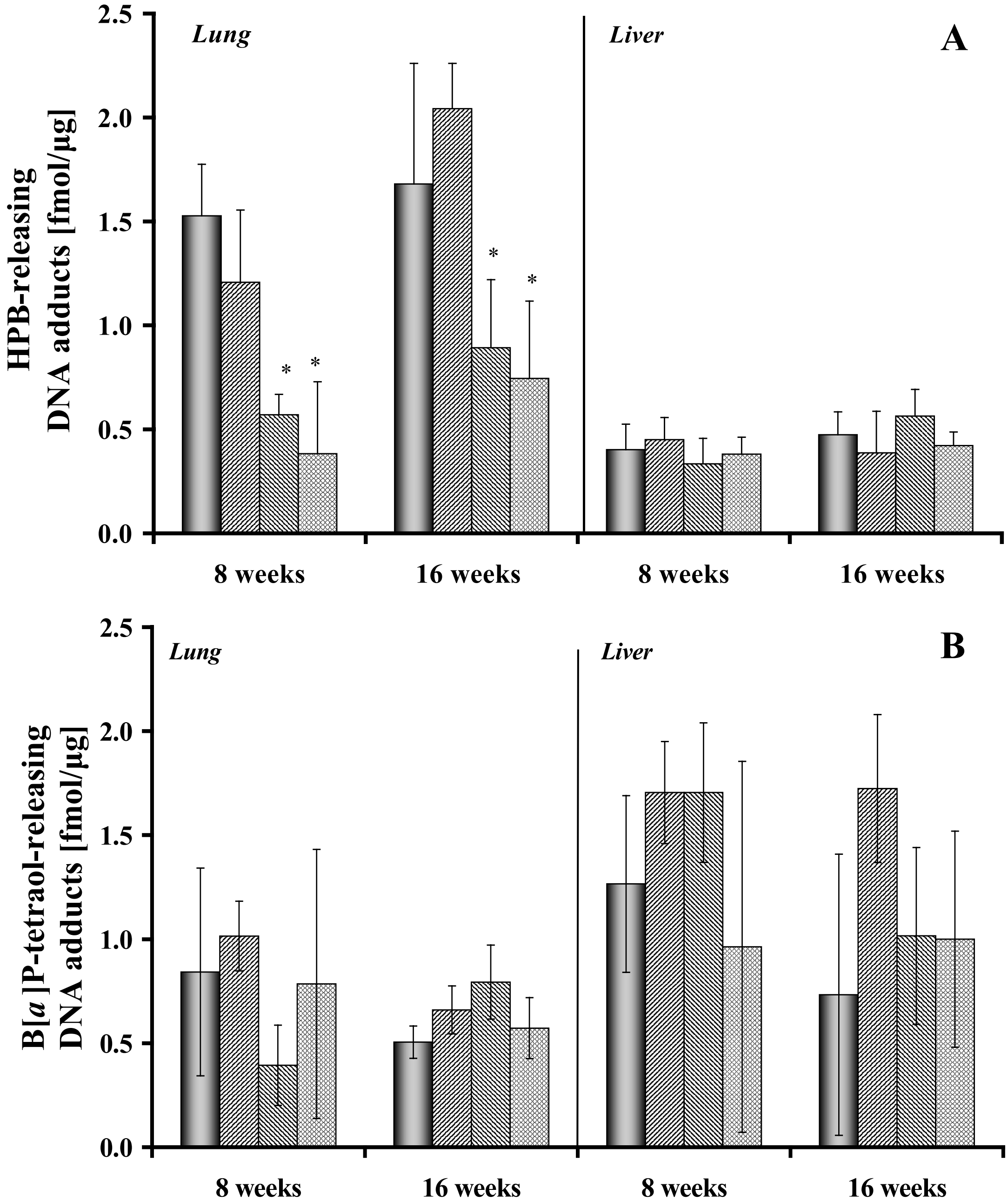

Figure 25: HPB-releasing (panel A) and B[*a*]P-tetraol-releasing (panel B) DNA adduct levels in lung and liver of rats treated with NNK (2 ppm) in the drinking water and B[*a*]P (2 ppm) in the diet ± PEITC (3µmol/g) and/or BITC (1µmol/g) in the diet (n=4). *,P<0.05.

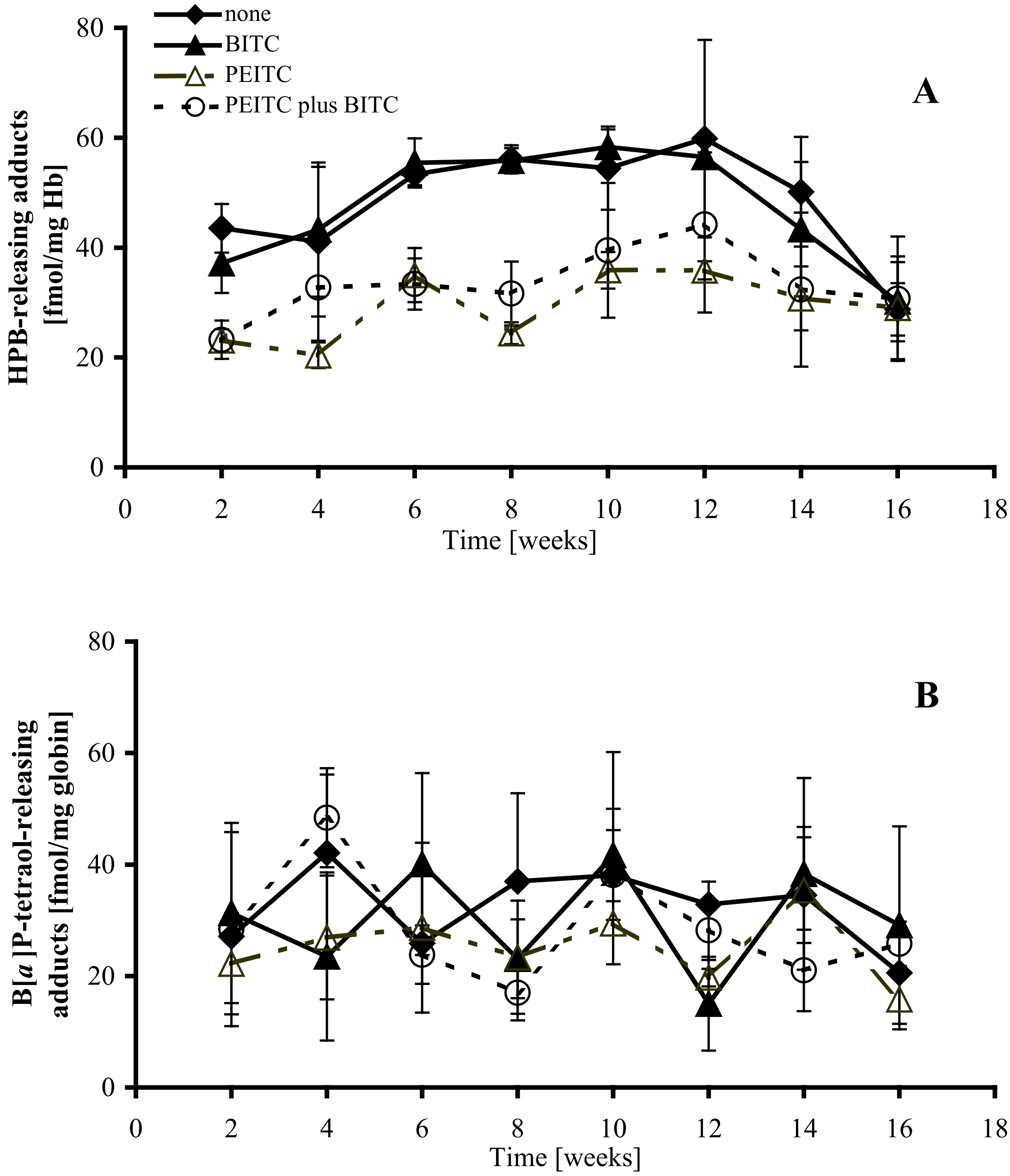

Figure 26: HPB-releasing-globin (panel A) and B[*a*]P-tetraol-releasing globin (panel B) globin adduct levels in rats treated with NNK (2 ppm) in the drinking water and B[*a*]P (2 ppm) in the diet ± PEITC (3μmol/g) and/or BITC (1 μmol/g) in the diet (n=4).

Table 15: DNA and globin adducts in F-344 rats treated with NNK plus B[*a*]P and ITCs

Group[a]	**Duration**	**Globin** [fmol/mg]	**Lung DNA** [fmol/µg]	**Liver DNA** [fmol/µg]
HPB-releasing adducts				
Control	week 8	56.1 ± 2.5	1.7 ± 0.6	0.5± 0.1
BITC		55.8 ± 2.3	1.3± 0.4	0.5± 0.1
PEITC		24.4 ± 2.0[b]	0.6 ± 0.1[b]	0.3 ± 0.1
PEITC plus BITC		31.7 ± 5.8[a]	0.5 ± 0.4[b]	0.4± 0.1
Control	week 16	29.8 ± 4.8	1.6± 0.3	0.4 ± 0.1
BITC		30.2 ± 7.2	1.7 ± 0.2	0.4± 0.2
PEITC		29.1 ± 9.3	0.4 ± 0.5[a]	0.6± 0.1
PEITC plus BITC		30.7 ± 11.3	0.8 ± 0.5[a]	0.4 ± 0.1
B[*a*]P-tetraol-releasing adducts				
Control	week 8	37 ± 15.8	0.5 ± 0.1	0.7 ± 0.7
BITC		23.1 ± 7.1	1.0± 0.3	1.4± 0.3
PEITC		23.4 ± 10.2	0.4± 0.2	1.7 ± 0.3
PEITC plus BITC		17.0 ± 5.0	0.8± 0.7	1.0 ± 0.9
Control	week 16	22.6 ± 6.7	0.8 ± 0.5	1.3± 0.4
BITC		29.1 ± 17.7	0.7± 0.1	1.7 ± 0.4
PEITC		15.8 ± 5.4	0.8± 0.2	1.0 ± 0.4
PEITC plus BITC		25.8 ± 4.0	0.6± 0.2	1.0 ± 0.5

[a]All rats were treated with NNK in the drinking water (2 ppm) and B[*a*]P in the diet (2 ppm). PEITC (3µmol /g diet) and BITC (1 µmol/g diet) were given in the diet.
[b] significantly different from control (P<0.05).

3.7 Effects of PEITC, PPITC and their *N*-acetylcysteine conjugates on adduct formation from NNN in F-344 rats

In animal study #4, the aim was to evaluate the effects of PEITC, PPITC and their *N*-acetyl conjugates on the formation of HPB-releasing adducts derived from NNN, a strong esophageal carcinogen. α-Hydroxylation of NNN at the 2′-position produces pyridyloxobutyl DNA adducts, which can be hydrolyzed to release HPB. The released HPB is then derivatized with PFBC and quantified by GC-NICI-MS. HPB-releasing-adducts were analyzed in esophageal DNA and hemoglobin by GC-NICI-MS.

HPB releasing DNA adduct in esophageal DNA of NNN treated rats

Pools of two esophagi giving a mean ± SD amount of 109 ± 27.4 µg DNA, were analyzed for HPB-releasing adducts. The mean ± SD recovery of the [$^2H_2$]HPB was 37 ± 12%. The mean background was 105 ± 10.6 fmol/sample (n=15) and was subtracted from each amount detected. HPB-releasing DNA adduct levels ranged from 171-981 fmol/µg DNA. The mean ± SD level detected was 351.7 ± 134.1 fmol/sample, which is about 3-fold above the background. The mean ± SD adduct level was 2.01 fmol/ µg DNA. One sample did not give any HPB-PFB or [$^2H_2$]HPB-PFB peak for technical reasons and one other had an exceptionally high adduct level. These data points were excluded with 95% confidence by statistical analysis (according to Q-test (164)). There were no differences in DNA adduct formation among the groups (Figure 27, Table 16).

HPB releasing Hb adducts in NNN treated rats

An average of 81.5 ± 43 mg Hb per sample was analyzed for HPB releasing adducts as described. The average recovery of the [$^2H_2$]HPB was 49 ± 33%. The mean background was 86.4 ± 42 fmol/sample (n=11) and was subtracted from each detected amount. The mean amount detected in the positive controls was 351.77 ± 42.5 fmol. The mean ± SD amount detected was 464.94 ± 302.4, and was about 4-fold above the background. The adduct levels were expressed as fmol HPB released (corrected for background)/mg hemoglobin. There were no effects of ITCs or their NAC conjugates on the formation of HPB-globin adduct levels (Figure 28, Table 16). Two samples had exceptionally high adduct levels and were excluded for statistical analysis with 95% confidence (according to the Q-test (164)).

Table 16: HPB-releasing DNA adduct levels in esophagus of NNN treated F-344 rats

Group[a]	**4 weeks mean ± SD**	**10 weeks mean ± SD**	**16 weeks mean ± SD**
Esophageal DNA adducts [fmol/µg DNA]			
NNN	1.78 ±0.50	2.14 ±1.04	1.68 ±0.63
NNN+PEITC	2.07 ±1.53[b]	2.43 ±1.07	1.15 ±0.32
NNN+PEITC-NAC	2.28 ±0.70	2.21 ±0.72	1.16 ±0.47
NNN+PPITC	1.97 ±1.01	2.07 ±0.61	2.11 ±1.30
NNN+PPITC-NAC	1.51 ±0.83	3.26 ±1.50	2.10 ±1.44
Hemoglobin adducts [fmol/mg HB]			
NNN	6.47 ± 1.5	5.05 ± 1.4	6.18 ± 1.6
NNN+PEITC	6.36 ± 1.3	5.15 ± 0.6	5.35 ± 2.0
NNN+PEITC-NAC	5.06 ± 0.7	6.18 ± 1.6	4.59 ± 1.2
NNN+PPITC	6.31 ± 1.9	7.14 ± 1.2[b]	4.76 ± 1.3[b]
NNN+PPITC-NAC	5.27 ± 1.0	7.17 ± 0.9[c]	6.20 ± 3.6

Data represent mean ± SD of 5 pools of two esophagi (n=5). NNN (5 ppm) was given in drinking water, ITC or their conjugates were given in the diet (1.0 µmol/g diet).
[a] One sample with no data and one with was excluded with CI 95% (n=4).
[b] One data point was excluded with CI 95% (n=4).
[c] One sample had no recovery for reason not known (n=4).

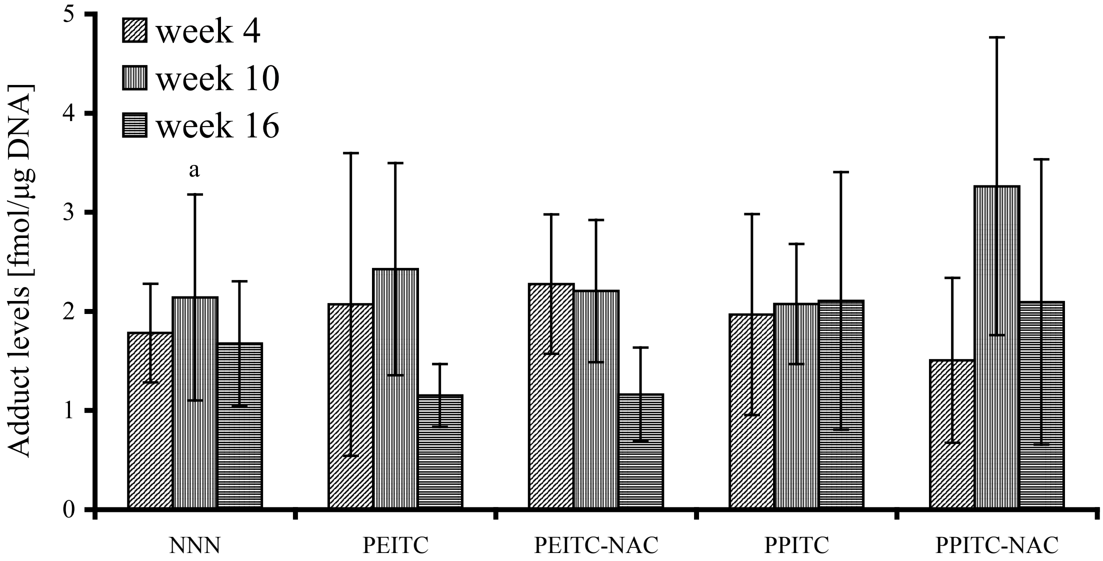

Figure 27: HPB-releasing adducts in esophageal DNA of rats treated with NNN (5 ppm) in drinking water and ITC or their conjugates in the diet (1.0 µmol/g diet).
[a] One data point was excluded with CI >90% (n=4).

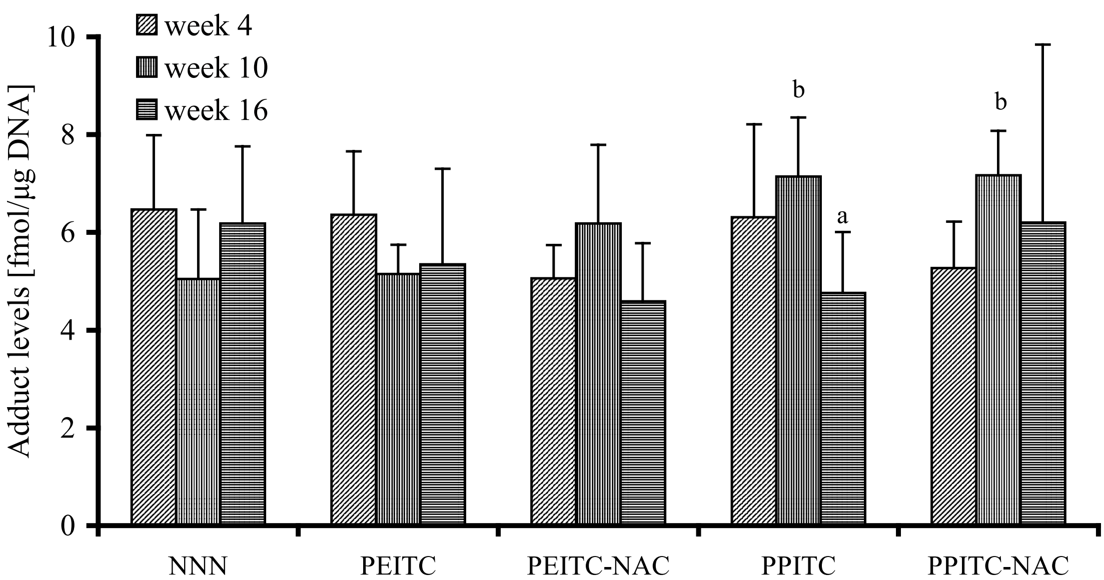

Figure 28: HPB-releasing hemoglobin adduct levels in rats treated with NNN (5 ppm) in drinking water and ITC or their conjugates in the diet (1.0 µmol/g diet).
[a] One with no data for technical reasons (n=4) .
[b] One data point was rejected with CI>95% according to Q-test (n=4) (164).
Figure 29

4 Discussion

4.1 Establishment of methods for the detection of BPDE-adducts

Initial experiments demonstrated that HPLC-FD and GC-MS are equally suitable for the detection of B[*a*]P-tetraol-releasing adducts. Depending on the nature of the study each has its advantages.

The HPLC-FD method was precise at levels as low as 5 fmol/ sample. It is easy to use and relatively inexpensive. While this method has been used in other studies on human exposures, we have for the first time determined its precision at different levels. Alexandrov et al. (108) tested the reproducibility of the HPLC-FD method at a level of 31 fmol/sample and found a variability of less than 3 %. Our results are in agreement, but we also demonstrated that at lower levels these CVs were 5%, 11.2% and 18.5% for 100, 25 and 5 fmol, respectively. The awareness of a level-dependent-standard error is important for determining slight differences between study groups at low levels, as discussed below. Analysis of globin by HPLC-FD results in multiple peaks eluting at similar retention times as the B[*a*]P-tetraols, creating an LOD of greater than 100 fmol/sample (data not shown).

The GC-NICI-MS method has greater absolute sensitivity, with an ability to quantify as low as 1 fmol/ standard on column. However, the LOD is 10-25 fmol/ sample, with a CV < 20 % at all levels determined, due to the complex work-up and recoveries of 10-40%. The larger variation is most likely due to the extensive sample preparation. An advantage of this method is its suitability for analysis of B[*a*]P-tetraol-releasing globin adducts, with the same LOD and precision (data not shown).

Both methods detect B[*a*]P-tetraols released upon mild acid hydrolysis and thus give limited information regarding the structure of the actual adduct. However they are more specific than the most commonly used techniques, ^{32}P-postlableling and immunoassay, because they detect a B[*a*]P-specific metabolite, the B[*a*]P-tetraol. In general, ^{32}P-postlabeling, with either thin layer chromatography or HPLC as separation systems provides neither the desired qualitative nor quantitative data. The specificity and selectivity of the immuno assay depends on the antibodies used, as they are known to cross-react with other PAHs. While studies in animals showed clear results (165-167), it is extremely difficult to retrieve specific data from human specimens (168,169), which are naturally exposed to a variety of compounds. Thus HPLC-FD and GC-NICI-MS are valuable tools in molecular epidemiological studies, and provide reliable results.

Surprisingly, the percentages of detected B[*a*]P-tetraols, by HPLC-FD or GC-NICI-MS range only from 33%-52% in human tissues (Boysen and Hecht in preparation (170)). It appears that no specific exposure leads to higher percentages of detected, or higher levels of B[*a*]P-tetraol-releasing adducts. An explanation may be that either the B[*a*]P-tetraol-releasing adducts are not present, or the levels are below the LOD of currently applied techniques.

Adduct levels detected by ^{32}P-Postlabeling or immuno assay usually ranging from 0.1–100 adducts/ 10^8nucleotides. ^{32}P-Postlabeling or immuno assay presumably measure mixtures of PAH-DNA and other possible DNA adducts suggesting that the levels of B[*a*]P-tetraol-releasing adducts are actually much lower. Alexandrov et al. (108) estimated that B[*a*]P-tetraol-releasing adducts may represent 10%–70% of total adducts measured by ^{32}P-postlabeling. Thus, the actual levels of B[*a*]P-tetraol-releasing adducts may be as low as 0.01–1.0 adducts/10^8 nucleotides. These levels are below the LOD of currently applied methods. More sensitive methods therefore are needed to reliably study B[*a*]P-tetraol-releasing adduct levels in humans and assess their contributions to cancer development and cancer risk.

4.2 Effects of PEITC and BITC on adduct formation from NNK plus B[*a*]P in A/J mice and F-344 rats

4.2.1 HPB-releasing adducts

Previous reports demonstrated the importance of DNA adduct formation in A/J mouse lung tumor induction (43,49,171). However, there are no reports in the literature on DNA adduct formation by mixtures of NNK plus B[*a*]P. The A/J mouse lung tumor model was ideal for assessing the effects of PEITC and BITC on DNA adduct formation from NNK plus B[*a*]P because effects of PEITC and BITC were already established (50,135).

The results presented herein support in part the hypothesis that the mechanism by which PEITC prevents NNK plus B[*a*]P-induced tumorigenesis involves the inhibition of DNA adduct formation. Dietary PEITC reduced the levels of HPB-releasing adducts by ~50% in lung DNA of A/J mice and F-344 rats treated with NNK plus B[*a*]P. These effects were observed whether or not BITC was present. This is consistent with previous findings that PEITC reduces HPB-releasing adducts derived from NNK in mice (42,43) and rats (48,172). However, O^6-mG and B[*a*]P-tetraol-releasing adducts were not affected by PEITC, BITC or both.

DNA adduct formation from NNK and B[a]P given alone or together

We first demonstrated that adduct levels in A/J mouse lung were not different when NNK and B[*a*]P were given individually or as a mixture, by gavage at levels known to induce lung tumors (Figure 20). The treatments applied herein were expected to produce about 3.3, 7.3 and 22.7 tumors per mouse in the groups treated for 1 ,4 or 8 weeks, respectively, based on total doses of NNK plus B[*a*]P (50). Thus, NNK and B[*a*]P do not effect the adduct formation of each other.

Effects of dietary PEITC and BITC on HPB-releasing DNA adducts in A/J mouse lung.

DNA adducts were analyzed in lung after treatments with NNK plus B[*a*]P, that are known to induce lung tumors (50), and with or without PEITC plus BITC treatments, that are known to inhibit NNK plus B[*a*]P-induced tumor multiplicity (135). Levels of HPB-releasing DNA adducts derived from NNK were significantly decreased in several groups treated with dietary PEITC or dietary PEITC plus BITC (Figure 24). It was in these groups that the strongest inhibition of lung tumor multiplicity was observed in a previous study (135). At the 24 h time point, a 30–40% inhibition of HPB-releasing DNA adducts was particularly consistent after 4 and 8 weeks of treatment. This inhibition correlates well with the inhibition of tumor multiplicity by ~40% at the same doses (135).

Interestingly, inhibition of HPB-releasing adduct levels was observed in all groups where the initial adduct levels were greater than ~1 fmol/μg DNA (Figure 18). This suggests that levels below 1 fmol/ μg DNA represent adducts derived from a PEITC-insensitive activation, while higher adduct levels are produced by a lung specific, PEITC-sensitive pathway. Since it is not clear if this effect is caused by one or multiple enzymes, the terms "PEITC-sensitive" and "PEITC-insensitive" pathway are used to describe either a single enzyme, presumably a P450, or a whole enzymatic pathway.

After one treatment of NNK plus B[*a*]P (3 μmol each), no effects of ITCs on HPB-releasing adduct levels were observed. Apparently adduct levels after one treatment, are not high enough to be affected by PEITC and/or BITC. This is in agreement with the observation described above that levels below 1 fmol/ μg DNA are produced by a PEITC-insensitive pathway. Previous reports state that a single dose of 2.5 μmol NNK (51) or 1 μmol NNK once weekly for 8 weeks (8 μmol total) do not significantly increase tumor multiplicity (50). However, 1 μmol NNK plus 1 μmol B[*a*]P once weekly for 8 weeks (total 8 μmol) produce a threefold increase in tumor multiplicity, mainly due to B[*a*]P rather than NNK (50).

Effects of gavaged PEITC and BITC on HPB-releasing DNA adducts in A/J mouse lung.

While dietary PEITC clearly inhibited the formation of HPB-releasing DNA adducts in lung, the effects of gavaged PEITC were weak and rarely significant. An inhibitory effect on the formation of adducts was observed in the groups treated for 4 or 8 weeks. In these groups, the reduction of HPB-releasing adduct levels was strongest after 8 weeks when receiving PEITC alone (Figure 21). Increased levels of BITC seemed to counteracted the inhibitory effect of PEITC, but due to large variations within the groups, most differences were not significant. This is consistent with the finding that dietary PEITC plus BITC were more effective inhibitors of NNK plus B[*a*]P-induced lung tumorigenesis than when they were given by gavage (31). However, the gavaged PEITC plus BITC treatments applied here are known to inhibit NNK plus B[*a*]P-induced tumor multiplicity by 30–35%. The different effects of dietary versus gavage treatments may be explained by the fact that the total PEITC dose is lower in the gavage groups (6–12 μmol PEITC) than in the dietary treatments, where each animal consumed about 42 μmol PEITC before the first carcinogen challenge. Mechanistically, Zhang and Talalay et al. demonstrated that ITCs accumulate rapidly to high (800–900 μM) intracellular levels in cell cultures exposed to low levels (1–5 μM) of ITCs (173). Thus, *in vivo* dietary ITC may produce intracellular concentrations that exceed those caused by gavage treatment, especially when the treatment are a week apart.

Effects of dietary PEITC and BITC on HPB-releasing DNA adducts in rat lung and liver

We then extended our investigation on HPB-releasing DNA adducts to F-344 rats treated with BITC and/or PEITC and a mixture of NNK plus B[*a*]P using a similar treatment protocol. The importance of HPB-releasing adducts in tumor formation in rat lung was demonstrated (172) (47). In spite of this, to our knowledge, no data are available on tumorigenesis and DNA adduct formation by mixtures of NNK plus B[*a*]P and their inhibition by PEITC or BITC in rats. Effects of PEITC and BITC on HPB-releasing DNA adduct levels in rat lung were similar those observed in mouse lung. Dietary PEITC reduced HPB-releasing DNA adducts by ~50% whether or not BITC was present. This is consistent with previous findings of PEITC-reduced HPB-releasing adducts in mice and rats (48,174) (Table 17).

Table 17: HPB-releasing DNA adducts in rat lung and liver

Treatment		dose[a]	HPB-releasing adducts[b] Lung	Liver	Lung/ liver	Reference
NNK	8 and 16 weeks	90-204	1.6[c]	0.5[c]	3.20	Present study
NNK+PEITC			0.6[c]	0.6[c]	1.0	
NNK	4 consecutive days i.p.	75	0.21	0.11	1.91	Murphy et al. (47)
		150	0.41	0.37	1.11	
		300	0.73	0.71	1.03	
		600	1.17	1.3	0.90	
		1200	1.64	4.4	0.37	
NNK	4 consecutive days s.c.	600	1.2	1.7	0.71	Morse et al.
NNK+PEITC			0.7	1.1	0.64	(174)
NNK	Once i.p.	2080	8.3	63.9	0.13	Peterson et al. (42)

[a] reported as µg NNK/kg/day.
[b] units = pmol/µmol guanine
[c] units = fmol/µg DNA

PEITC did not effect the HPB-releasing DNA adduct levels in the liver and levels were about half of that in lung. In this study adduct levels were higher in lung than in liver. Murphy et al. reported similarly higher levels in lung than in liver (47). However, this was only observed for doses below 600 µg/kg/day of NNK (significant for doses of 75 µg/kg/day or less) while at higher doses adduct levels were higher in liver than in lung. This suggests an important change in the metabolic activation/detoxification of NNK, dependent on the dose of NNK. The treatments herein corresponded to about 90-204 µg/kg/day for 8 or 16 weeks (low doses). Despite the longer treatment time, by generating a higher total dose of NNK plus B[*a*]P, the HBP-releasing adducts in lung remained higher than in liver (Figure 25). The ratio was even higher than expected, based on data from Murphy et al. (47) (Table 17). This suggests that chronic exposure can lead to the accumulation of adducts. Thus NNK may be a much stronger lung carcinogen at a lower level of exposure than expected. In contrast to our findings Morse et al. showed that PEITC (3 µmol/g diet) starting 11 days prior to 4 daily subcutaneous (s.c.) injections of 600 µg NNK, reduced HPB-releasing adducts by 42% in lung and by 35% in liver

(174). A possible explanation may be that high doses of NNK, as applied by Morse et al., induces or enhances NNK-activation in liver. This NNK-induced or -enhanced metabolism is PEITC-sensitive. In lung however it seems α-methyl hydroxylation of NNK is not affected by NNK dose and is PEITC-sensitive at all doses of NNK.

An interesting observation is that dietary PEITC reduced HPB-releasing DNA adduct levels in lung to similar levels as those observed in liver. It seems that HPB-releasing DNA adduct levels of <1.0 fmol HPB/μg DNA, as seen in liver, represent PEITC-insensitive activation of NNK, while higher DNA adduct levels of >1.0 fmol/ μg DNA in lung are PEITC-sensitive. This is consistent with results observed in the A/J mouse lung and may be a key observation of this study.

Similar effects of dietary PEITC on NNK-activation as well as NNK-induced or NNK-enhanced activation were observed in lung and liver microsome preparations from mice and rats. The influence of pre-treatment with NNK or NNK+PEITC on the metabolic activation of NNK via α-hydroxylation was explored in rat microsomes (175). NNK was given by s.c. (1.76mg/kg body weight) weekly for 4, 12, or 20 weeks, and PEITC was given in the diet (3 μmol/g diet). When compared to NNK pretreated groups, PEITC significantly inhibited formation of the α-hydroxylation products, HPB and keto aldehyde, in lung microsomes from rats pretreated with NNK plus PEITC. Liver microsomes from the same animals were unaffected by pretreatment with NNK or NNK plus PEITC (175). Even more interesting is that PEITC-pretreatment reduced the rate of HPB and keto aldehyde formation in lung microsomes to the rates reported for the liver microsomes from the same animals. This is consistent with our observation that PEITC reduces the HPB-releasing DNA adduct levels in lung to levels measured in liver. Comparable results have been reported for mouse lung microsome preparations (176). Pretreatment with 3 μmol PEITC/g diet for 4 weeks resulted in a 40% and 30% reduced formation of HPB and keto aldehyde, respectively. Liver microsomes from mice pretreated with PEITC formation of HPB and keto aldehyde were 20% and 30% reduced (176). The NNK concentration was higher (10 μM NNK) in the mouse microsome preparations than in the rat microsome preparations (3 μM), suggesting that NNK induces or enhances its own metabolic activation in liver. These results may explain the increased formation of HPB-releasing adducts reported by Murphy et al. (47). PEITC acts as a non-competitive and competitive inhibitor, as shown by decreasing the V_{max} value but does not influence the K_m in mice lung and liver microsomes incubated with 10 µmol NNK (176,177).

Extensive studies have clearly shown that α-hydroxylation of NNK is catalyzed by multiple P450s (31). In rat and mouse lung α-hydroxylation involves P4502B1 and 2A enzymes, or immunochemically related forms. P450 2B1 has consistently shown to catalyze α-methylene hydroxylation (178,179), while 2A enzymes seem to be responsible for α-methylene and α-methyl hydroxylation in lung (180). Antibodies against rat 1A1, 2B1 and 2A1 inhibit HPB formation in mouse lung (176,177), however 1A1 is not detected in mouse lung (180), suggesting that some P450s, important for NNK activation in lung, have not been characterized. Felicia et al. recently showed that P450 2A5 is important in NNK activation in mouse lung (181). Further, human P450 2A13 was highly effective in metabolic activation of NNK, as reported by Su et al. (182). The kinetics of α-hydroxylation is in general biphasic (177). Devereux et al. demonstrated *in vivo* that formation of O^6-mG in rat lung cells is more efficient at lower dose of NNK than at higher doses (178). In rat and mouse lung, antibodies against P450 2B1 inhibit α-methylene-hydroxylation of 10 µmol NNK by 23% (176,180) while concentrations of 20 µmol NNK are inhibited by 45% (179) (Table 18). Several P450s appear to play a minor role in NNK activation. Inhibition of 1A1, 2E1 and 2C11 had no effect on α-methylene or α-methyl hydroxylation of NNK in rodents (176,179,180,183).

Table 18: Inhibition of methylation by P450 specific antibodies

α-Methylene-hydroxylation[a]		**P450**			**Reference**
		1A2	2A1	2B1	
Rat lung	10 µmol NNK	46%	64%	23%	(180)
	20 µmol NNK			32%	(179)
Mouse lung	10 µmol NNK	9%	47%	23%	(176)
	20 µmol NNK			45%	(179)

[a] Thus far data on α-methyl-hydroxylation in rodents are only done at 10 µmol NNK

We therefore conclude that in rodents exist at least two separate activation pathways for NNK. One is highly lung specific and can be inhibited by PEITC (PEITC-sensitive), leading to elevated HPB-releasing adduct levels in lung. The other, PEITC-insensitve, is more general and gives rise to relatively low levels of HPB-releasing adducts in lung. A high doses of NNK, on the other hand, can induce or enhance the PEITC-sensitive pathway in the liver. The PEITC-sensitive pathway may be permanently expressed in lung while its inducible in the liver.

Effects of PEITC and BITC on HPB-releasing hemoglobin adducts in F-344 rats

The formation of HPB-releasing hemoglobin adducts was reduced 50% by dietary PEITC over the first 12 weeks. This is consistent with previous reports where HPB-Hb adducts were ~50% lower in rats treated with dietary PEITC (143). The HPB-releasing Hb adduct levels were half of those reported in an earlier study using the same doses of NNK and PEITC, but no B[*a*]P or BITC. After 12 weeks, HPB-releasing-Hb adduct levels declined in the control and BITC groups, suggesting that NNK plus B[*a*]P treatment may inhibit α-hydroxylation of NNK. Similar results were also observed in a previous report (143), but statistical analysis was not performed.

The time course of HPB-releasing-Hb adducts did not correlate with adduct formation in the lung or liver. The degrees of inhibition of Hb-adducts was similar to that in lung at 8 weeks, but not at 16 weeks. In liver, no effect of PEITC or PEITC plus BITC was observed. These results suggest that there may be a tissue other then lung or liver that specifically activates NNK, leading to HPB-releasing-Hb adducts. This activation site can be inhibited by PEITC or PEITC plus BITC (PEITC-sensitive) and is also inhibited by NNK plus B[*a*]P treatment after 16 weeks of treatment. It is interesting that the formation of O^6-mG in lung of F-344 rats treated with NNK (1.76 mg/kg three times weekly) and dietary PEITC (3μmol g/ diet) follows a similar pattern (48). In that study, HPB-releasing adducts in whole lung were ~40-50% inhibited by PEITC over 20 weeks. O^6-mG levels remained almost steady in the PEITC-treated group, while the levels in the NNK only group were increased between 8 and 12 weeks and declined after 12 weeks. These are exactly the effects we observed for the HPB-releasing-Hb adducts. Thus far, we have no explanation that would link the formation O^6-mG in lung to the formation of HPB-releasing-Hb adducts.

The results of the long term dietary PEITC on different endpoints in NNK carcinogenesis in mice and rats are summarized in Figure 30. It clearly shows that the effects of PEITC are similar for each endpoint but different between mouse and rat. A linear correlation is found along the endpoints, demonstrating their suitability. The slope is not null because of the protective effects of cellular defense systems (e.g. DNA repair). The activation of NNK to a pyridyloxobutyldiazohydroxide does not always leads to tumor development; it may undergo glucuronidation and be excreted (39) or decompose to HPB. Furthermore, only a small percentage of HPB-releasing adducts cause mutations leading to tumors. Most are either repaired by the DNA repair system or do not cause mutations in critical genes. HPB-releasing hemoglobin adducts are not mechanistically involved in the carcinogenesis and therefore represent a surrogate exposure marker. From Figure 30 one can estimate that in lung the PEITC-sensitive pathway contributes α-methyl hydroxylation of NNK by ~50% in mice and 90% in rats. Future studies of these biomarkers in humans may help to predict the efficacy of PEITC and other chemopreventive agents in humans.

In summary, PEITC is known to inhibit NNK-induced lung tumorigenesis and microsomal activation in rodents. We demonstrated herein that the formation of HPB-releasing DNA adducts is inhibited to a similar extent. The remaining tumorigenicity, microsomal activation of NNK, and formation of HPB-releasing adducts, must be caused by the PEITC-insensitive pathway. It is therefore concluded that the tumorigenicity of NNK is due to at least two activation pathways, of which one may be completely blocked by PEITC while the other is not.

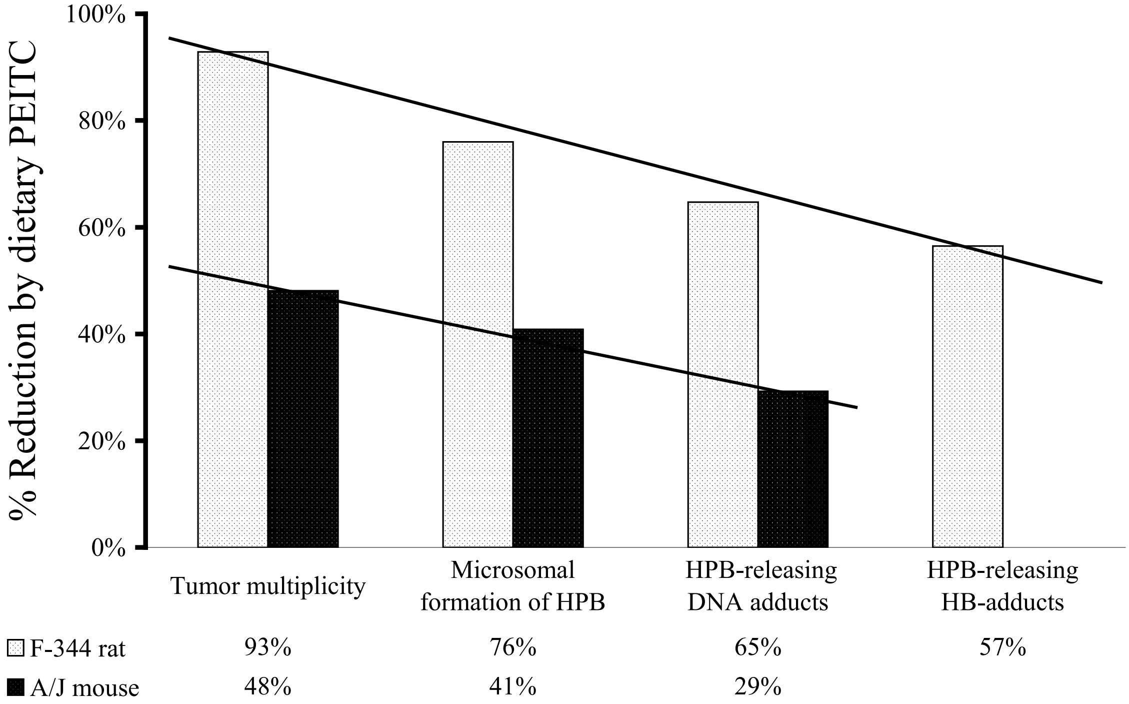

Figure 30: Effects of dietary PEITC on different biological endpoints in NNK carcinogenesis.
Tumor multiplicity data are from references (143,184).
Microsomal formation of HPB are from references (175,176).
DNA and Hb adduct data are from present study.

4.2.2 O^6m-G adducts from NNK plus B[*a*]P

The null effect of PEITC and BITC treatments, both dietary and by gavage, on O^6-mG levels was surprising. Previous studies demonstrated convincingly that O^6-mG is important in lung tumorigenesis in A/J mice treated with a single dose of NNK, and that PEITC inhibit O^6-mG adduct levels. (43,173,184). Morse et al. treated mice for four consecutive days with 5 or 25 µmol of PEITC by gavage, corresponding to a total of 20 and 100 µmol PEITC. This was followed by 10 µmol of NNK by i.p. injection (184). Levels of O^6-mG, 2 h and 6 h after NNK administration, were significantly reduced by PEITC. In a second study, four consecutive gavage doses of 1 µmol PEITC, 2 h prior to 10 µmol NNK by gavage had no influence on O^6-mG levels in mice 6 h later (173). In the study by Morse et al., inhibition of O^6-mG levels was observed at total doses of PEITC that were two or ten times higher than that of NNK. In the study presented here, the PEITC dose after a single treatment was two or four times that of NNK, but no inhibition was seen. Surprisingly, after 4 or 8 weekly gavage treatments, resulting in even higher doses of PEITC, no change was observed. Dietary treatments of about 6 µmol PEITC/ day starting one week before the administration of NNK plus B[*a*]P had no effects. One explanation may be that B[*a*]P, prevents the PEITC-mediated inhibition of O^6-mG levels. While B[*a*]P does not affect the O^6-mG levels directly, it may compete in some way in some way with NNK for the active site in the enzyme responsible for α-methylene hydroxylation, thereby preventing inhibition by PEITC.

Alternatively, the different doses of NNK (10 µmol by Morse et al. versus 3 µmol once a week in our study) may affect the proportion of PEITC-sensitive to PEITC-insensitive pathways for α-methylene hydroxylation, similar to α-methyl hydroxylation, as discussed above. Thus, the level given by Morse et al. may lead to PEITC-sensitive formation of O^6-mG, while our chronic treatment with lower levels of NNK do not.

4.2.3 B[*a*]P-tetraol releasing adducts

Effects of gavaged PEITC and BITC on B[a]P-tetraol–releasing adducts in A/J mice

While dietary ITC administered by gavage had little or no effect on DNA adduct levels resulting from NNK, we observed consistently lower levels of B[*a*]P-tetraol-releasing adducts in the groups treated by gavage with PEITC plus BITC (Figure 23). This was significant in most of the groups sacrificed 120 h after the last NNK plus B[*a*]P treatment. This may result from induction of nucleotide excision repair (NER). Huang et al. demonstrated that PEITC induces p53 expression (185). Furthermore, cells that lack p53 are deficient in removal of B[*a*]P-tetraols releasing adducts (186). These data suggest that PEITC, and perhaps BITC as well, may induce expression of NER genes by a p53-mediated pathway causing an inhibition in the formation of B[*a*]P-tetraol adducts detected at the later time points. The inhibition of B[*a*]P-tetraol-releasing adducts, however, is consistent only in part with the results of our tumor induction study, where we observed inhibition only at the higher combinations of PEITC plus BITC (135). Effects of ITC other than modification of DNA adducts may therefore be involved in inhibition of tumorigenesis. These potential effects are discussed further below.

The extent of inhibition was somewhat similar to those reported by Sticha et al. in a time-course study (187). PEITC given by gavage (6 µmol in 0.2 ml cottonseed oil) or in diet (3 µmol/ g diet) reduced B[*a*]P-tetraol-releasing adduct levels by 40% over a time period of 2–298 h after the last treatment of NNK plus B[*a*]P by gavage (3 µmol each in 0.2 ml cottonseed oil) (187). Administration of PEITC plus BITC had a similar effect on B[*a*]P-tetraol-releasing adducts. However, lung tumor multiplicity in B[*a*]P-treated A/J mice was inhibited by BITC and not by PEITC (144,145,188). We Therefore conclude that there are mechanisms other than inhibition of adduct formation responsible for inhibition of lung carcinogenesis by BITC.

Effects of dietary PEITC and BITC on B[a]P-tetraol-releasing DNA adducts in lung of A/J mice and F-344 rats

In contrast to the results of the gavaged PEITC and BITC treatments in mice, no influence on B[*a*]P-tetraol-releasing DNA adduct levels was observed in any of the groups receiving dietary BITC, PEITC, or PEITC plus BITC (Figure 24C). These results are consistent with previous tumor inhibition studies, which suggested that tumor inhibition by dietary PEITC plus BITC is due to the influence of PEITC on NNK-induced tumors (135). The bolus dose of PEITC and BITC by gavage had markedly different consequences than the gradual relatively low dose of dietary administration. There may be toxic effects associated with the gavage administration, although there was no significant change in animal weights in our study or in previous work by Hecht et al. (135).

The levels of DNA adducts derived from B[*a*]P in the lung of F-344 rats were not affected by treatment with dietary BITC and/or PEITC at the doses given in this study (Figure 25B). This is consistent with results in A/J mice, where dietary treatment with BITC (1µmol/g diet) and/or PEITC (3 µmol/g diet) did not affect the B[*a*]P-tetraol-releasing DNA adduct levels in the lung when NNK plus B[*a*]P were given by gavage.

Effects of dietary PEITC and BITC on B[a]P-tetraol-releasing in liver DNA and globin addutcs in F-344 rats

The treatments with PEITC and BITC were also ineffective on B[*a*]P-tetraol-releasing DNA adducts in the liver of F-344 rats. Consistent with previous findings, that the liver is the main tissue of B[*a*]P metabolism, adduct levels were higher in liver than in lung. Similar to observations in lung and liver, the treatments with PEITC or BITC had no effect on B[*a*]P-tetraol-releasing globin adduct levels. This findings is not surprising because globin adducts are believed to represent the effects of metabolic activation mainly in liver. Thus, there was no effects on DNA adduct levels, hence one would not expect effects on globin adduct levels.

Anderson and co-worker reported that in rats the total binding of B[*a*]P to DNA is about twice as high in liver than in lung (189). Conversely, the BPDE-derived (B[*a*]P-tetraol releasing) adducts were not detected in liver. The main adduct detected by Anderson et al. in liver of rats was tentatively identified as being B[*a*]P-phenol-derived (189) and is presumably the recently identified 5,9-dihydroxy-4,5-dihydro-5-(N^2-guanosyl)-benzo[*a*]pyrene adduct (190). This discrepancy may be explained by the different treatment protocols. Boroujerdi et al. gave a single intravenous injection of 1 or 10 µmol B[*a*]P and sacrificed the rats 1 h later. We explored chronic exposure through the diet (0.063 µmol/day). Further, it is most likely that the sacrifice time by Boroujerdi et al. may have been to short to produce significant amounts of B[*a*]P-tetraol-releasing adducts. In contrast to our results in rats, Stowers et al. reported similar adduct levels, mainly B[*a*]P-tetraol-releasing, between lung and liver in mice and rabbits (191), 24 and 48 h after B[*a*]P treatment (191). Thus, metabolic activation of B[*a*]P in rats may give rise to different adducts than the B[*a*]P-tetraol-releasing adducts, and these adducts may be important, too. Due to the specificity of the GC-NICI-MS method applied herein, we were not able to observe adducts other than the ones releasing *trans/anti* B[*a*]P tetraol. In liver DNA from rats treated with 100 mg or 400 mg B[*a*]P i.p., we observed two adducts by HPLC-FD, that were distinctly different from the B[*a*]P-tetraol-releasing adducts and were not detected in the control animals (data not shown). The structure of this adducts were not further investigated at this point.

4.3 Effect of PEITC and PPITC and their NAC-conjugates on adduct formation from NNN

In the last part of this study, we used NNN as carcinogen and PEITC and PPITC and their NAC-conjugates as inhibitor to determine whether the prevention of esophageal tumors is due to inhibition of adduct formation (Figure 27 and Figure 28). We did not observe any effects of the ITCs tested on the formation of HPB-releasing DNA adducts in esophagus or globin adducts. This was surprising because the protocol used herein is known to produce a 71% tumor incidence and PPITC was shown to significantly inhibit the tumor incidence (72). Additionally, PPITC was reported to significantly reduce the formation of hydroxy acid and keto acid in esophageal explants. The formation of these acids is an indication of α-hydroxylation, expected to produce HPB-releasing DNA *in vivo* (64) and *in vitro* (45). Furthermore, NNN is shown to produce HPB-releasing globin adducts in rats (68). Consequently, our results clearly showed that the prevention of esophageal tumorigenesis by PEICT, PPITC and their NAC-conjugates must be a result of a mechanism other than inhibition of HPB-releasing adducts.

ITCs were shown to effectively inhibit NMBA-induced esophageal tumorigenesis (192-194). Fong and co-worker demonstrated that the induction of esophageal tumors by NMBA can be prevented by rapid induced apoptosis (195). In a zinc-deficient (ZD) rat model a single dose was highly tumorigenic (196), that was otherwise nontumorigenic (197). In addition it was demonstrated that when a zinc-sufficient diet was administrated after NMBA treatments, tumor incidence was reduced from 100% in ZD rats to 14% in zinc-repelished rats (195,198). The zinc repelished tissues had rapid increase in the apoptotic index (198). This clearly shows that the ability to induce apoptosis efficiently is important in tumorigenesis. Thus, PPITC and other ITCs might increase the apoptotic response (see also chapter 4.4).

4.4 Other effects of isothiocyanates

The results discussed above indicate that there are additional mechanisms of ITCs other than modification of DNA adduct formation. Prominent among these is the induction of apoptosis (152). A considerable body of evidence indicates that the induction of apoptosis is important in chemoprevention by ITCs.

D'Agostini et al. demonstrated that PEITC increases cigarette smoke-induced apoptosis in the respiratory tract of rats (199). Huang et al. found that PEITC induces apoptosis in mouse epidermal JB6 cells through a p53-dependent pathway (185), and furthermore, PEITC induces apoptosis in human leukemia cells, as shown by Xu et al. (200). Induction of apoptosis by BITC and sulforaphane was observed in human colon cancer cells (201-204).

Multiple studies on mechanisms of ITC–induced apoptosis have consistently shown involvement of increased caspase-3(-like) activity (205-207) leading to the proteolytic cleavage of poly(ADP-ribose) and DNA fragmentation (205). Other work identified the involvement of c-Jun-N-terminal kinases activation (206), activation of MAP kinase, expression of AP-1 transcription factor, p53 phosphorylation (208) and the mitochondrial death pathway (207).

Yang et al. investigated the effects of BITC-NAC and PEITC-NAC on molecular events associated with apoptosis in B[*a*]P–treated A/J mice (208). Both ITCs inhibited B[*a*]P-induced tumorigenesis. The MAP kinase pathway was activated in the ITC-NAC-treated groups. The activation of c-Jun N-terminal kinase was higher in the BITC-NAC and PEITC-NAC groups when compared with B[*a*]P-treated control. The phosphorylation of p38 and extracellular signal-regulated kinase (ErKs) 1 and 2 was also induced by these treatments. The AP-1-binding activity was remarkably increased in lung tissue from both the BITC-NAC and PEITC-NAC groups. Phosphorylation of p53 was also higher than the constitutive levels in both ITC-NAC-treated groups, but no induction of p53 expression was detected. (208).

4.5 Limitations of studies presented herein

There were some limitations to these studies. First, we examined only a few specific DNA adducts. While there is substantial evidence that the adducts measured are important in carcinogenesis by NNK plus B[*a*]P, it is possible that other adducts, not measured here, may also contribute to tumorigenesis.

To investigate DNA adducts derived from NNK, we measured HPB-releasing adducts as a representative of α-methyl-hydroxylation, however individual pyridyloxobutyl adducts, such as N^2- or O^6-[4-oxo-4-(3-pyridyl)butyl]deoxoguanosine (209) may be important in carcinogenesis. In mice we also analyzed O^6-mG derived from α-methylene hydroxylation of NNK, but O^4-mT and 7-mG and other unidentified NNK derived adducts may also be involved in tumorigenesis (40). While 2'-hydroxylation of NNN produces HPB-releasing adducts, several other unidentified adducts have been observed (45). Little is know about the fate of the diazohydroxide from 5'-hydroxylation of NNN. 5'-hydroxyl-NNN is structurally related to the α-hydroxy-*N*-nitrosopyrrolidine and 4-oxobutane 1-diazohydroxide, formed upon α-hydroxylation of *N*-nitrosopyrrolidine. Adducts from this intermediate have been extensively characterized (210). In addition, B[*a*]P is know to depurinate DNA (211) and produce 7-(benzo[*a*]pyren-6-yl)guanine and 7-(benzo[*a*]pyren-6-yl)adenine adducts (212). Recently, a 5,9-dihydroxy-4,5-dihydro-5-(N^2-guanosyl)-benzo[*a*]pyrene adduct has been identified in rat microsomal incubations (190).

Furthermore, all measurements were carried out in whole tissue (either lung or liver), but it is possible that adducts in individual cell types may be formed differently, thus obscuring the overall effects. Staretz et al. demonstrated that the time course of O^6-mG and HPB-releasing adducts in NNK treated rats is significantly different among various cell types (e.g. Clara cells, type II cells small cells (48)).

In summary, the results presented here partly support the hypothesis that the modification of DNA adduct levels is involved in inhibition of NNK plus B[*a*]P-induced lung carcinogenesis by ITCs. Effects other than inhibition of adduct formation are most likely contribute to chemoprevention by ITCs.

4.6 Future directions

It is long known that there are multiple enzymes or pathways for α-hydroxylation of NNK. We showed here that PEITC is a selective inhibitor for one of these pathways. Future studies on NNK activation therefore should include PEITC treatment to separate the PEITC-sensitive from PEITC-insensitive pathway. PEITC may be helpful in identifying P450s responsible for NNK activation in lung (PEITC-sensitive). It will also be important to identify additional chemopreventive compounds that inhibit the PEITC-insensitive pathway so that it compliments the PEITC-treatment.

There is some evidence that in liver, NNK may enhance its own activation at higher doses or concentrations. This should be kept in mind when designing future studies. Previously, most studies used only one dose or concentration of NNK, and results were sometimes conflicting. Future studies should therefore always include high and low levels of NNK treatment.

We investigated only a limited number of DNA adducts and it may be possible the other adducts, not assayed herein, and may be important. Therefore, future efforts should be made to identify other relevant DNA adducts of NNK, NNN and B[*a*]P. Our group is currently working on the identification of the HPB-releasing adduct or adducts.

According to our working hypothesis, the next step would be to assay whether ITCs reduce mutation frequencies *in vivo*. Transgene systems are available to measure the mutation frequency in target tissues and may be useful to answer question regarding the effects of ITCs on mutagenesis.

Much research has already been conducted on induction of apoptosis. The correlation between markers for carcinogen exposure and apoptosis needs to be determined. This would enhance the overall picture of carcinogenesis.

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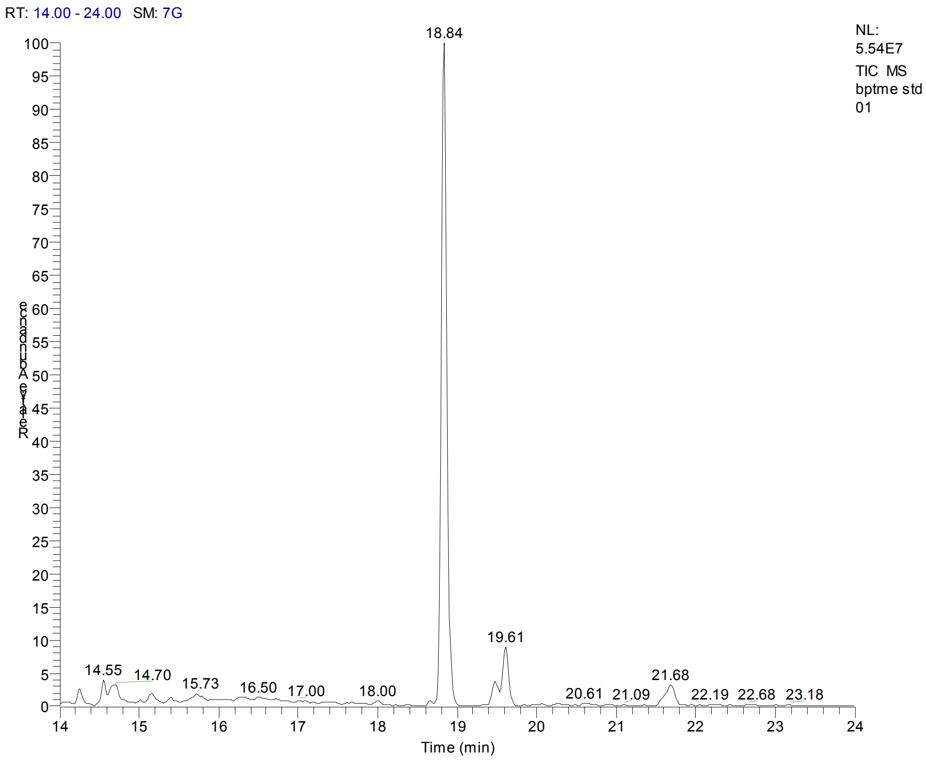
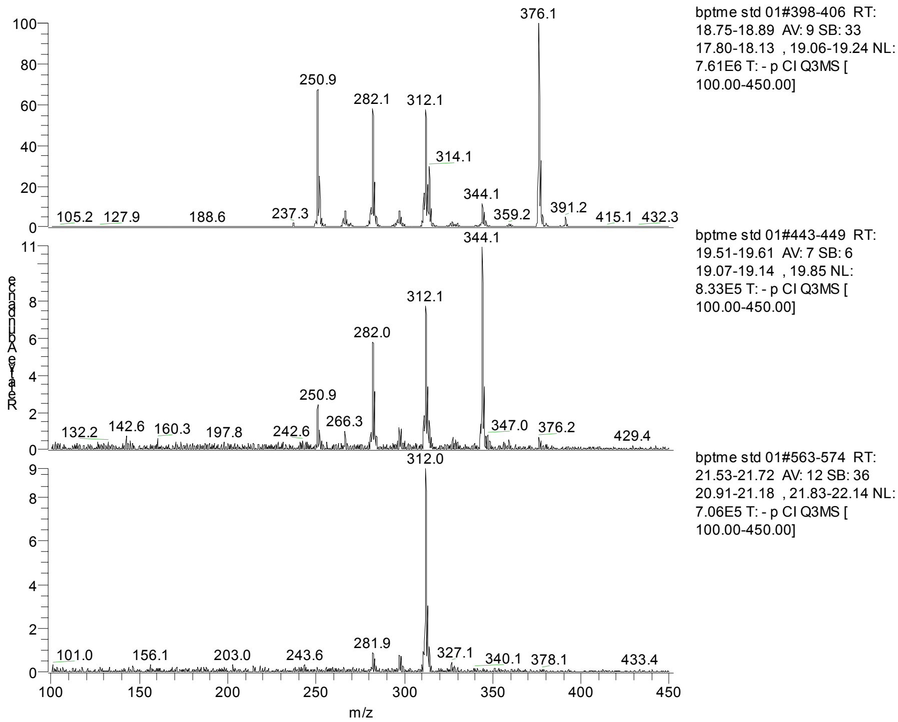
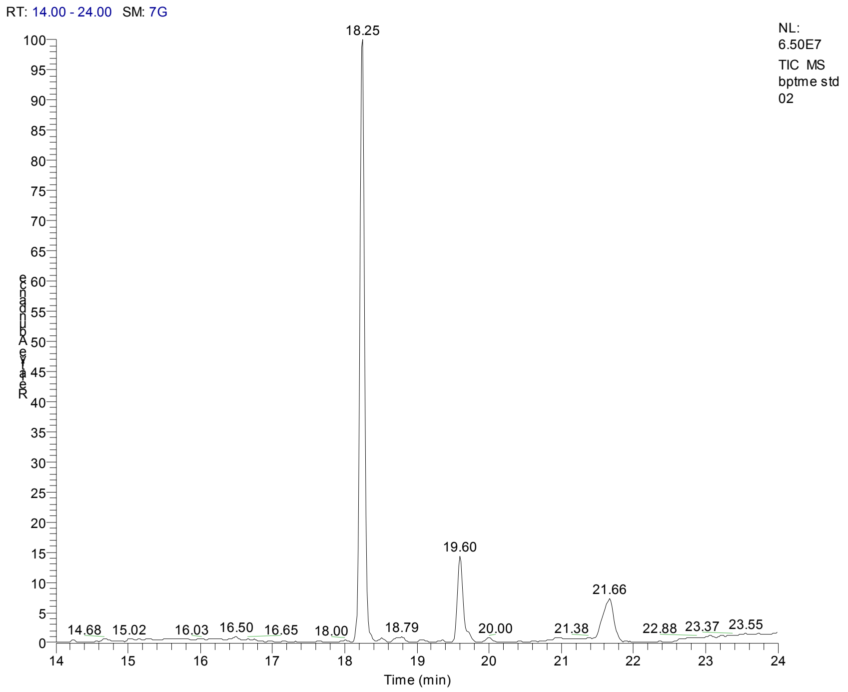
6 Appendix

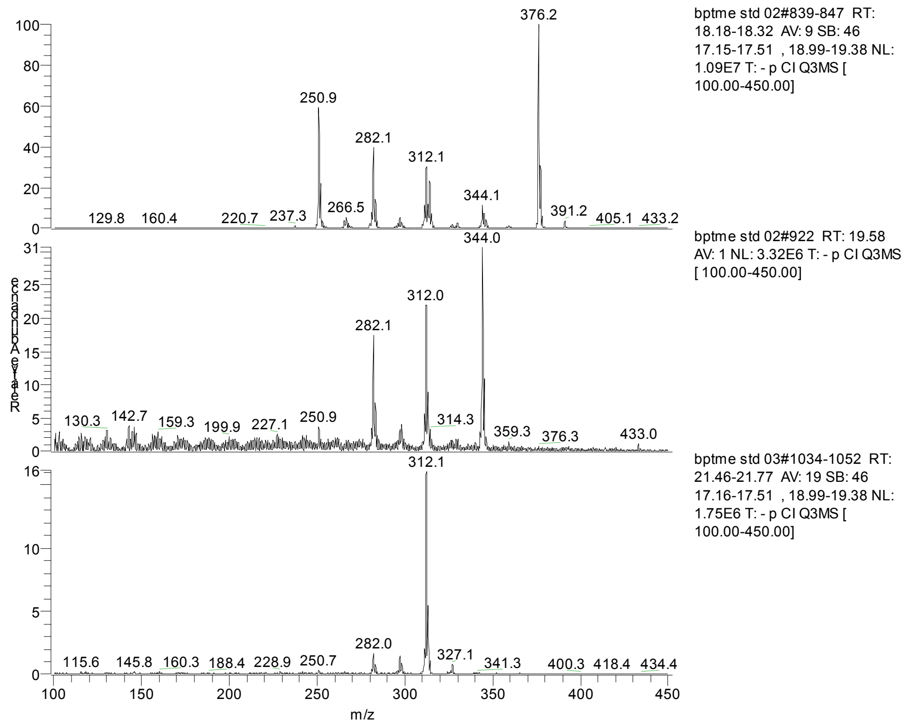
6.1 *trans/anti* B[*a*]P-TME



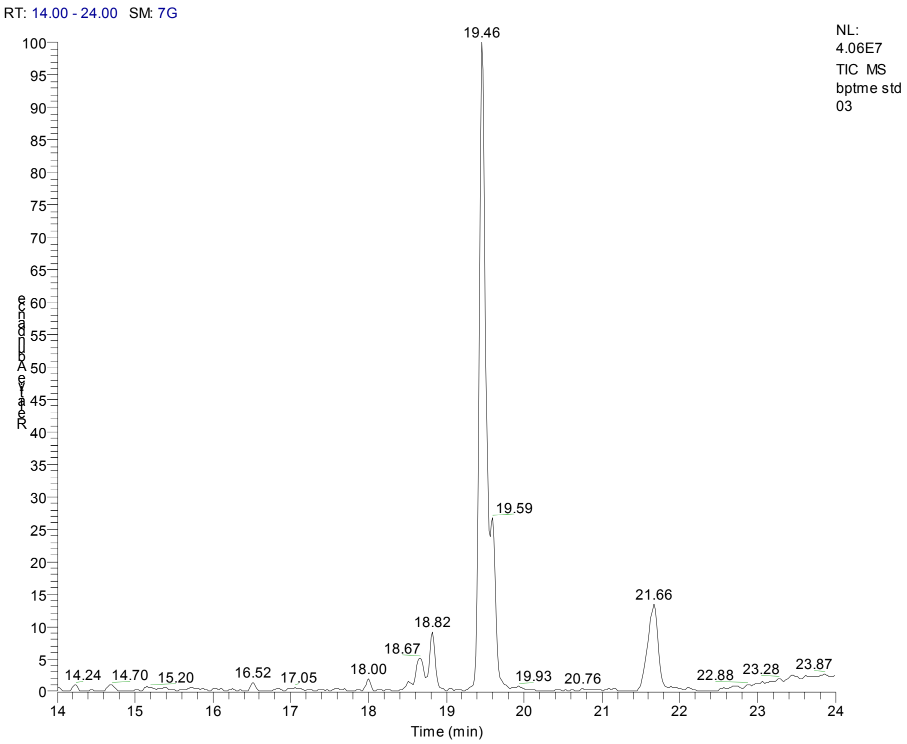


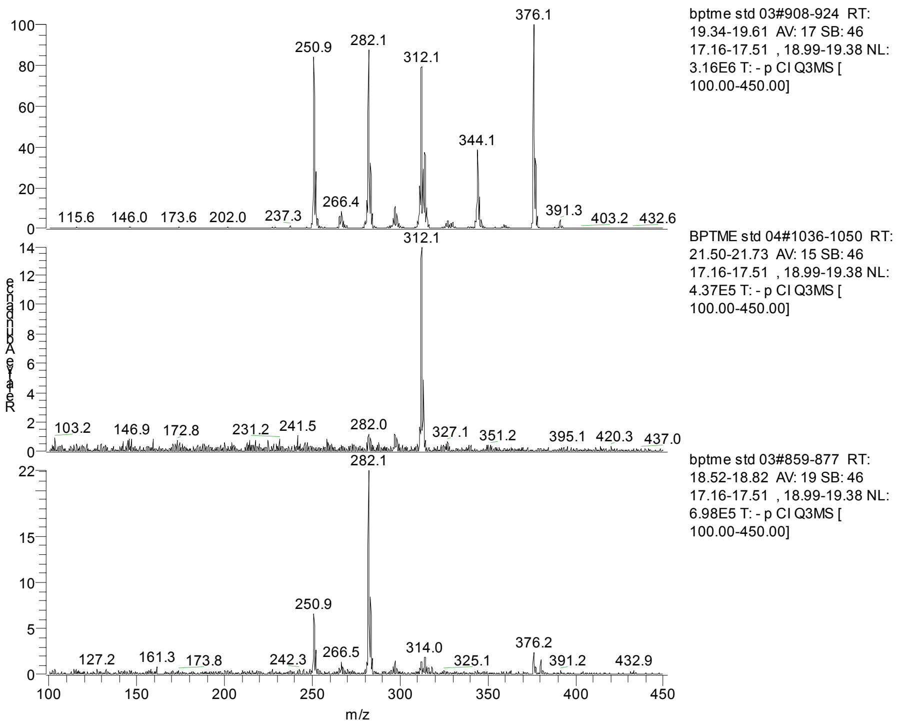
6.2 *trans/syn* B[*a*]P-TME



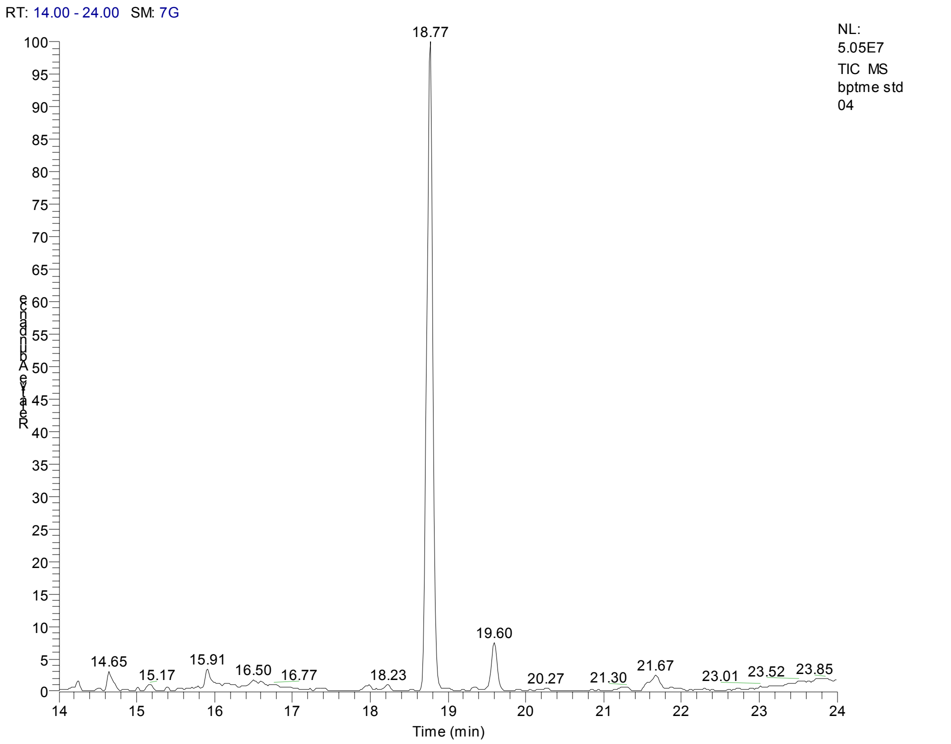


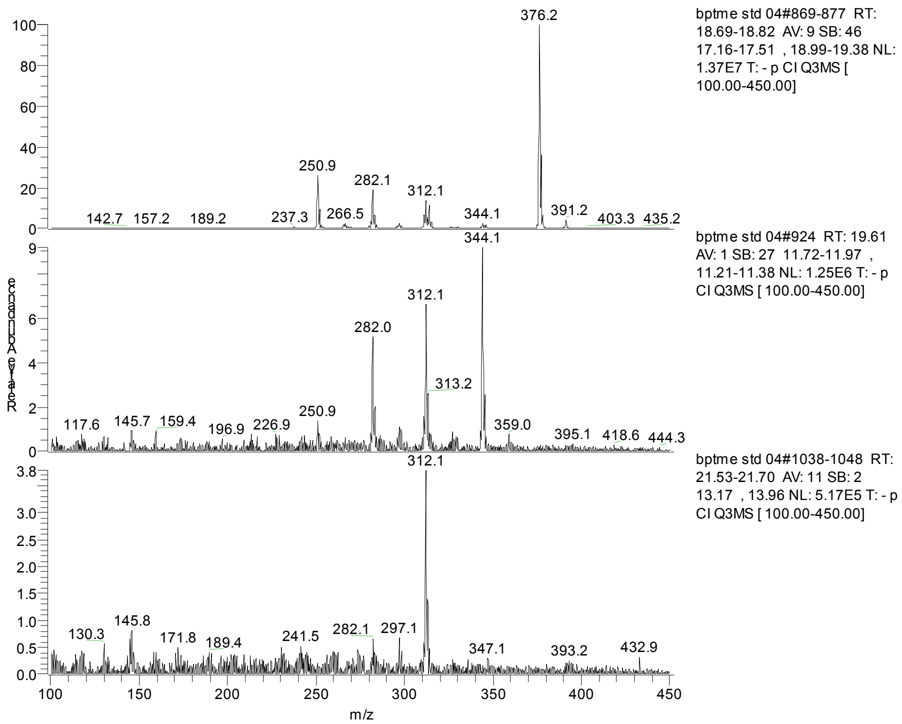
6.3 *cis/anti* B[*a*]P-TME



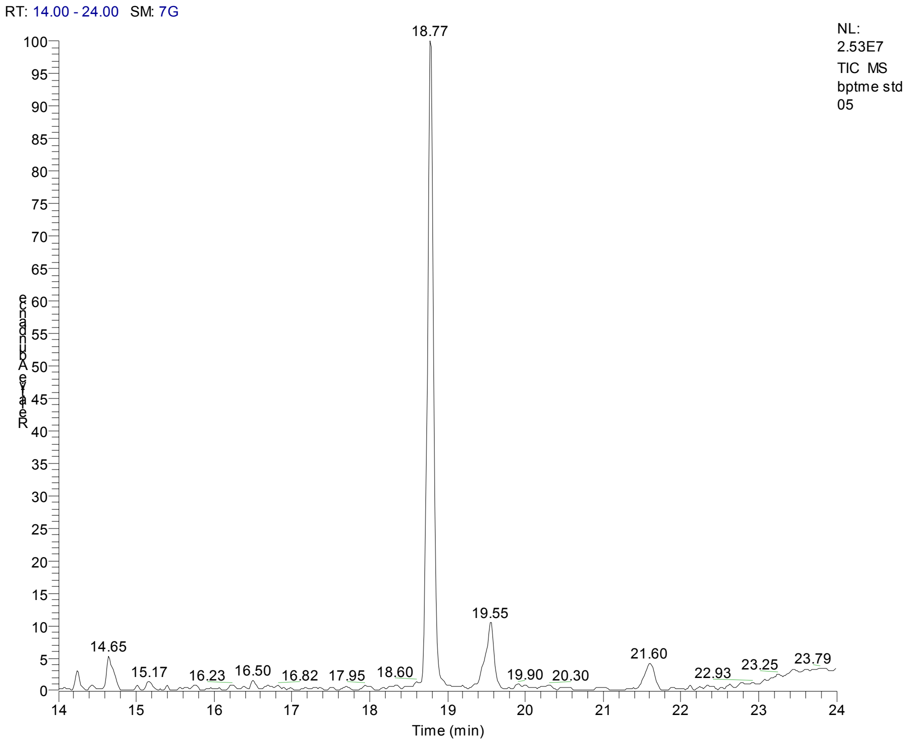


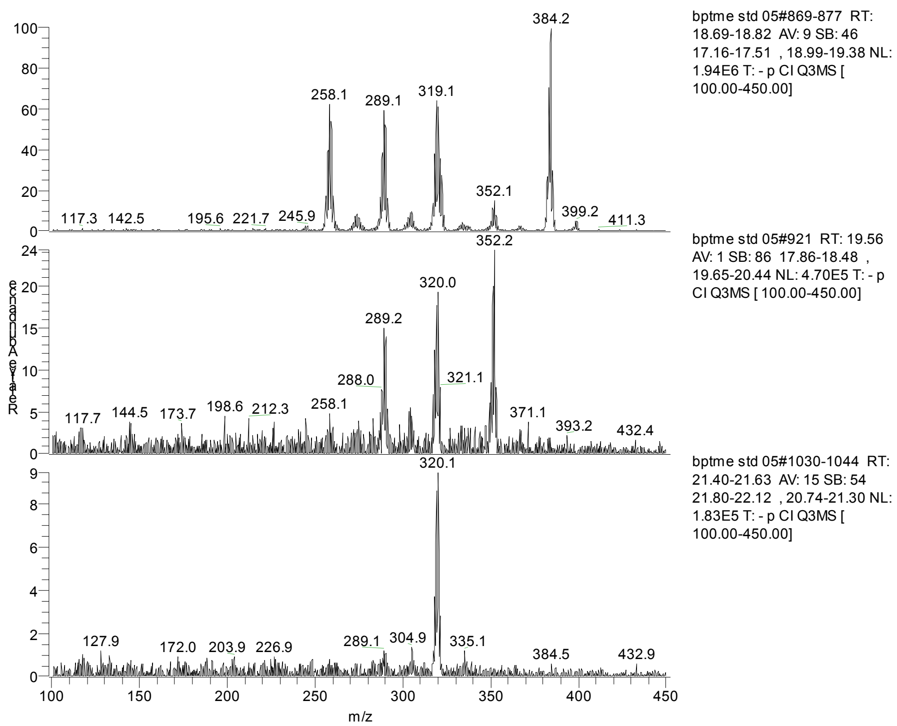
6.4 *cis/syn* B[*a*]P-TME



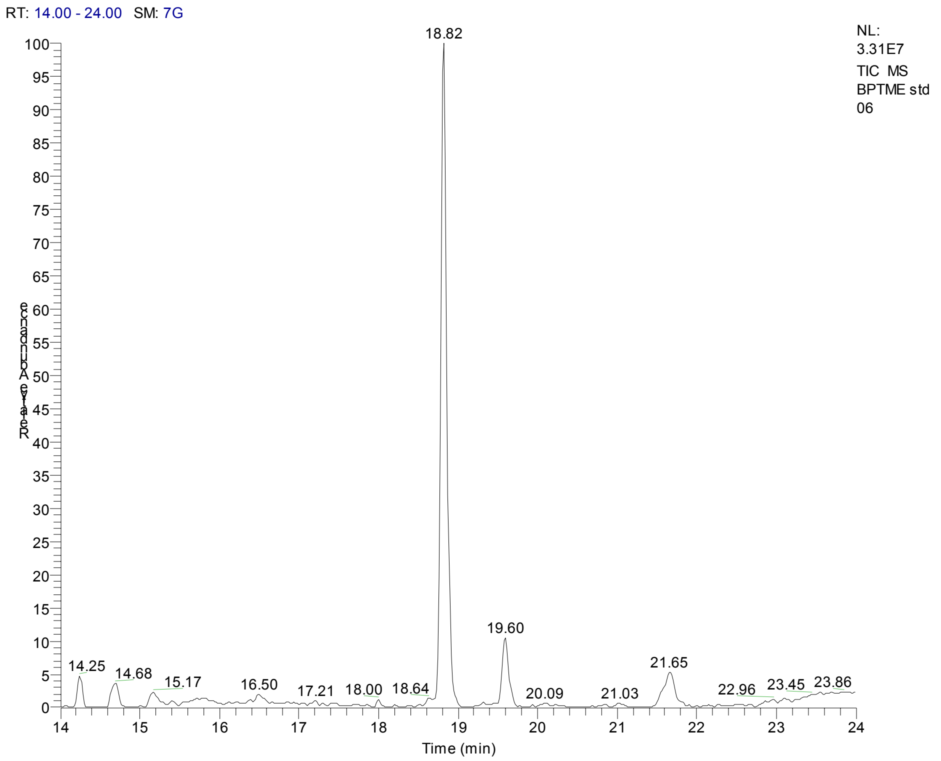


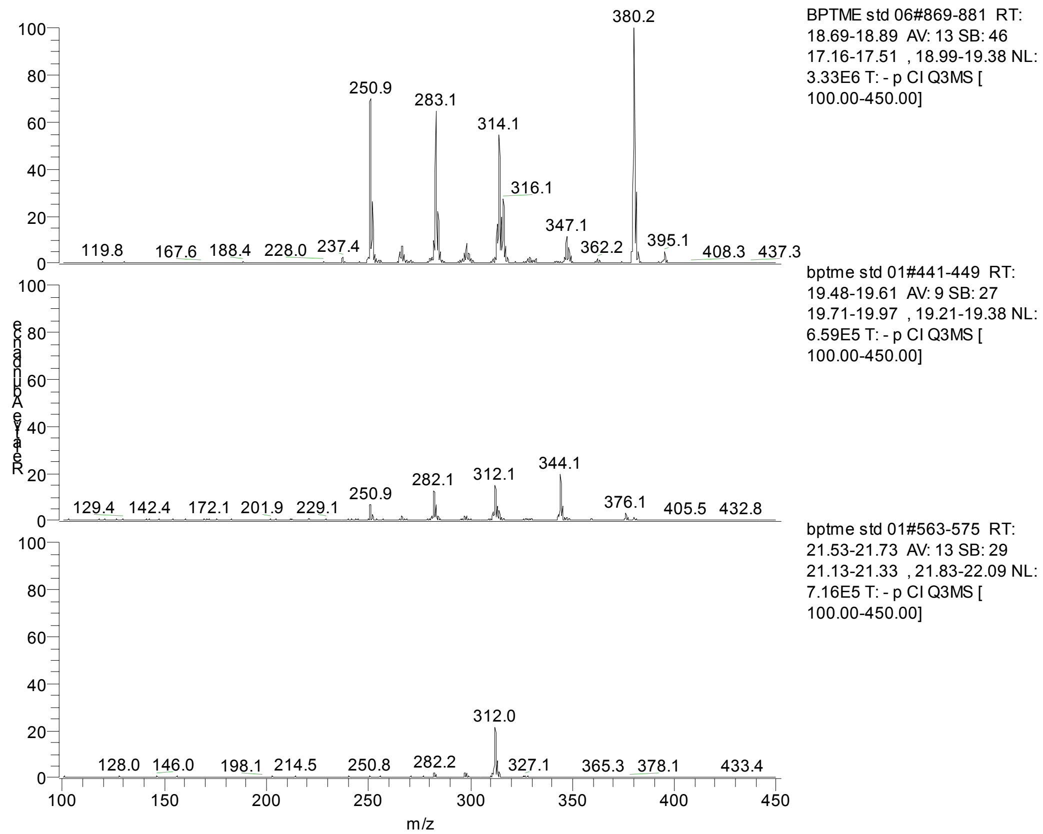
6.5 *trans/anti* [$^2H_8$]B[*a*]P-TME





6.6 *trans/anti* [$^{13}C_4$]B[*a*]P-TME





Curriculum Vitae

Name:	Gunnar Boysen
Born:	Dec. 30^{th} 1969, Flensburg, Germany
Nationality:	German

Education

Primary School	Aug. 1976 - July 1981
Elementary School Aug.	Aug. 1981 - July 1986
Technical High School and College Aug.	Aug. 1986 - July 1991
University of Kaiserslautern, Faculty of Biology	Aug. 1991 – Sep. 1996

Diploma:	Genetics	September 1996
	Human biology	
	Plant physiology	
	Biochemistry	

Professional experience:

Nov. 1995- Dec. 1996

Member of working group ‘Genetic Toxicology ‘
(head: Dr. P. Schmezer), Division of Toxicology and Cancer Risk Factor (head: Prof. Dr. H. Bartsch) at the German Cancer Research Center, Heidelberg.
In vivo mutation assay with transgenic animals
“comet assay“ for studying DNA damage
PCR based DNA sequencing
Southern and Northern Blotting
Cell culture techniques

Nov. 1995 – Sep. 1996

Diploma thesis: "Mutagenity of Cadmium salts in transgenic systems"

Jan. 1997 -present

Member of group of Prof. Dr. Hecht
University of Minnesota Cancer Center
Analysis of tobacco specific DNA adducts
Intense training in mass spectroscopy methods
(LC-MS and GC-MS)

Awards and Fellowships

Apr. 1997 - Mach 1998 Deutscher Akademischer Austauschdienst (DAAD)
(German Academic Exchange Service)
Doktorandenstipendium HSP II (Ph.D.-research grant)

Publications

Sticha,K.R.; Kenney,P.M.; **Boysen,G**.; Liang,H.; Su,X.; Wang,M.; Upadhyaya,P.; Hecht,S.S. (2002)
Effects of Benzyl Isothiocyanate and Phenethyl Isothiocyanate on DNA Adduct Formation by a Mixture of Benzo[*a*]pyrene and 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone in A/J mouse Lung. *Carcinogenesis* 2002 in press.

Boysen, G, Kenney, P. M. J., Wang, M., Upadhyay, P. and Hecht, S.S. (2002)
Effects of Phenethyl Isothiocyanate (PEITC) and/or Benzyl Isothiocyanate, on the Formation of DNA and Hemoglobin Adducts in F344 Rats Treated with 4-(Methyl-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) plus Benzo[*a*]pyrene (B[*a*]P). *Carcinogenesis*, *in preparation*

Boysen, G and Hecht S.S (2002)
Analysis of DNA and Protein Adducts of Benzo[*a*]pyrene in Human Tissues Using Structure-Specific Methods. *Muta. Res., accepted*

Boysen, G., Villalta P.W., Carmella S.G. and Hecht S.S. (2002)
Comparison of HPLC-FD, GC-MS and LC-MS methods for the detection of B[*a*]PDE-DNA adducts. *in preparation*

Poster presentations

Boysen, **G.**, Sticha, K., , Villalta P.W., Carmella S.G. and Hecht S.S. (1999)
Comparison of HPLC-FD, GC-MS and LC-MS methods for the detection of B[*a*]PDE-DNA adducts. 2nd *Annual Spring Symposium at University Minnesota Cancer Center*

Boysen, G. and Hecht S.S. (2001)
Analysis of DNA and Protein Adducts of Benzo[*a*]pyrene in Human Tissues Using Structure-Specific Methods: A review. *Transdisciplinary Tobacco Use Research Meeting.*

Boysen, G, Kenney, P. M. J., Wang, M., Upadhyay, P. and Hecht, S.S.
Effects of Phenethyl Isothiocyante (PEITC) and/or Benzyl Isothiocyanate, on the Formation of DNA and Hemoglobin Adducts in F344 Rats Treated with 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) plus Benzo[*a*]pyrene (B[*a*]P). *American Association for Cancer Research 93rd Annual Meeting 2002*