Effect of Peroxidase Inhibitors on an in Vivo Metabolite of the Urinary Bladder Carcinogen N-[4-(5-Nitro-2-furyl)-2-thiazolyl]formamide in Rats¹

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ABSTRACT

Peroxidase metabolism of 2-amino-4-(5-nitro-2-furyl)thiazole (ANFT) was evaluated in vitro and in vivo. In vitro metabolism of ANFT was characteristic of the hydroperoxidase activity of prostaglandin H synthase. The peroxidase inhibitors, 6-aminopropyl-2-thioracil and methimazole, significantly reduced ANFT binding to trichloroacetic acid precipitable material and glutathione conjugate formation. Isolated perfused kidneys rapidly converted the glutathione conjugate to its corresponding mercapturic acid (ANFT-MA). With both radiochemical and electrochemical techniques, ANFT-MA was identified in the urine of rats given N⁴[4-(5-nitro-2-furyl)-2-thiazolyl]formamide, the carcinogenic N-formyl analogue of ANFT. ANFT-MA was the major urinary metabolite with N⁴[4-(5-nitro-2-furyl)-2-thiazolyl]formamide not detected. A 30-min pretreatment with 6-aminopropyl-2-thioracil and methimazole significantly reduced urinary excretion of ANFT-MA in rats given N⁴[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (150 mg/kg) from 14.8 ± 2.1 (SE) to 7.9 ± 0.8 and 6.2 ± 1.1 mmol/18 h, respectively. Peroxidase inhibitor pretreatment did not alter the excretion of ANFT or prostaglandin E₂. These results provide further in vitro and in vivo support for the involvement of peroxidases, i.e., the hydroperoxidase activity of prostaglandin H synthase, in ANFT metabolism.

INTRODUCTION

Determination of enzymes involved in the initiation of chemically induced cancer will provide a better understanding of the carcinogenic process. An in vitro index of initiation is thought to be the binding of carcinogen to DNA (1-4). Certain aromatic and heterocyclic amine bladder carcinogens activated by PHS bind DNA (5-7). PHS consists of two distinct enzymatic activities, fatty acid cyclooxygenase and prostaglandin hydroperoxidase (8). Fatty acid cyclooxygenase catalyzes the biosynthesis of arachidonic acid to the cyclic endoperoxide PGG₂. Prostaglandin hydroperoxidase is responsible for the reduction of PGG₂. During this reduction, prostaglandin hydroperoxidase will oxidize suitable cosubstrates, i.e., aromatic and heterocyclic amines (9, 10). In this manner, the hydroperoxidase activity of PHS activates certain amines to electrophiles which bind DNA. Thus, this suggests a potential role for PHS in the genesis of malignancies by certain carcinogenic amines. However, only a few in vivo investigations have supported this hypothesis.

PHS catalyzed binding of 2-naphthylamine to DNA in vitro results in the formation of adducts derived from 2-amino-1-naphthol and N-hydroxy-2-naphthylamine (11). While the former adducts are specific for PHS, the latter may also be produced by mixed-function oxidase metabolism (12). Both 2-amino-1-naphthol and N-hydroxy-2-naphthylamine adducts were detected in dog bladder epithelium following a p.o. dose of 2-naphthylamine (11). These results are consistent with PHS involvement in the initiation of 2-naphthylamine induced bladder cancer. However, the exact contribution of each enzymatic pathway to adduct formation is difficult to assess because N-hydroxy adducts could be formed by both metabolic pathways. The FANFT/ANFT rat model for bladder cancer has also provided evidence for the involvement of PHS in initiation (13). By aspirin inhibiting fatty acid cyclooxygenase catalyzed peroxide formation (14), hydroperoxidase oxidation of amines is prevented (9). Aspirin reduces the incidence of early morphological bladder lesions (15) as well as the bladder tumors observed following administration of FANFT (the formylated analogue of the proximate carcinogen ANFT) (16). A unique renal metabolic/excretory coupling is responsible for the high levels of ANFT and the undetectable amounts of FANFT observed in urine following FANFT administration (17, 18). ANFT is susceptible to nitroreduction by several enzymatic systems (19-21). However, only one pathway for oxidative metabolism of ANFT has been reported (22). ANFT is oxidized by PHS but not by other peroxidases. ANFT is not oxidized by NADPH supplemented liver microsomal preparations which contain mixed-function oxidases and other oxidative enzymes (21). These unique features of the FANFT/ANFT model were used in the present study to provide further in vivo support for the involvement of PHS in the initiation of carcinogenesis.

MATERIALS AND METHODS

Animals and Materials. Male Fischer 344 rats (180-230 g, 6-8 weeks old) were obtained from HarLAN Sprague-Dawley, Inc., Indianapolis, IN. Unlabeled ANFT was obtained from SaBer Chemical Co., Morton Grove, IL. 2[¹⁴C]ANFT was synthesized from [¹⁴C]thiouria (New England Nuclear, Boston, MA) and FANFT was synthesized from ANFT by N-formylation as reported previously (23, 24). ANFT-SG and ANFT-MA were synthesized as reported previously (25). Glutathione, N-acetylcysteine, PTU, MMI, and 1-chloro-2,4-dinitrobenzene were obtained from Sigma Chemical Co., St. Louis, MO. Arachidonic acid was purchased from Nu-Chek Prep., Inc., Elysian, MN. High purity methanol and water were obtained from American Scientific Products, St. Louis, MO, for use as chromatographic mobile phase components.

In Vitro Studies. Standard incubations of microsomal protein with 2[¹⁴C]ANFT were performed at ambient temperature for 2 min and analyzed as reported previously (22, 25). Briefly, reaction mixtures contained solubilized ram seminal gland microsomal protein (0.11 mg) as a source of PHS, 100 mM phosphate buffer (pH 7.8), 0.001 mM hematin, and 0.02 mM ANFT and were initiated by the addition of 0.11 mM arachidonic acid in a total volume of 0.25 ml. Incubations to assess ANFT-SG formation included 0.25 mM glutathione. Binding of ANFT to protein was assessed following ethyl acetate extraction and subsequent precipitation of protein with TCA (6, 22). For determination of ANFT-SG, 0.01 ml of the reaction mixture was injected directly into the HPLC without further sample treatment. ANFT-SG was deter-

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⁴ The abbreviations used are: PHS, prostaglandin H synthase; ANFT, 2-amino-4-(5-nitro-2-furyl)thiazole; ANFT-MA, the thiazole ring-substituted mercapturic acid conjugate of ANFT; N⁴[4-(5-nitro-2-furyl)-2-thiazolyl]formamide; HPLC, high pressure liquid chromatography; MMI, methimazole, 2-mercapto-1-methylimidazole; PG, prostaglandin; PTU, 6-aminopropyl-2-thioracil; TCA, trichloroacetic acid.
mained by electrochemical detection. Arachidonic acid mediated metabolism of ANFT is expressed as nmol of product formed in the presence of arachidonic acid with values obtained in the absence of arachidonic acid subtracted. The latter values were similar to those obtained in the presence of indomethacin. As illustrated in Fig. 2, Panel 2, ANFT-SG levels were determined by change in absorbance at 340 nm (27) and ANFT-SG formation was examined as described for the whole blood studies. Rabbit liver glutathione S-transferase (Sigma) was also utilized to demonstrate the formation of ANFT-SG. To examine renal conversion of ANFT-SG to ANFT-MA, kidney slices were prepared for ex vivo perfusion as reported previously (17) and perfused for 120 min with 4.4 ± 0.1 μM ANFT-SG (initial concentration). Urine and perfusate samples were collected at timed intervals and were analyzed for ANFT-SG, ANFT-MA, insulin, sodium, and potassium (17, 25).

In Vivo Studies. Male rats (300–500 g) were anesthetized i.m. with ketamine-acepromazine and prepared for collection of bile and urine (18). In brief, the bladder was cannulated with PE-50 polyethylene tubing (Clay Adams, Parsippany, NJ) through a midline abdominal incision and the duodenum distal to the bile duct was ligated, thus allowing bile to accumulate in the stomach. Following collection of a 20-min blank urine sample, 1 μCi of 2-[^14C]FANFT plus 10 μmol of unlabeled FANFT in 5 ml of saline was infused i.v. via a cannulated right internal jugular vein. Urine was collected as 4 consecutive 30-min samples. After 2 h, the stomach was removed intact and the bile was drained out. Urine and bile were assayed for ANFT-MA and/or ANFT-SG by liquid chromatography as described below.

For the in vivo studies of the effect of PTU and MMI on FANFT disposition, individual rats were lightly anesthetized with diethyl ether and given 250 mg/kg of PTU or 167.5 mg/kg of MMI (1.5 mmol/kg) p.o. dissolved in dimethyl sulfoxide (500 μl/kg) by means of an 18-gauge stainless steel feeding tube (Popper and Sons, Inc., New Hyde Park, NY). This dose of PTU was approximately the minimum amount necessary to achieve inhibition of ANFT-MA excretion. Control rats were given only an equivalent dose of dimethyl sulfoxide. After 30 min, the rats were again lightly anesthetized and similarly dosed with 150 mg/kg FANFT (0.63 mmol/kg) dissolved in dimethyl sulfoxide (2.0 ml/kg). The dose of FANFT utilized appeared to have a slight sedative effect upon some of the animals but was otherwise well tolerated. Each animal was individually placed in a clean metabolic cage (Nalgene, Rochester, NY) which provided for separate collection of urine and feces with free access to food and water. Urine was collected for 18 h to ensure uniform sample volumes. At random times during the course of the study, individual rats were treated with equivalent doses of dimethyl sulfoxide not containing inhibitor or FANFT and their urine was used as a blank to ensure the integrity of the analyses for ANFT and ANFT-MA. Each animal was used for only one experiment. Following measurement of urine volume, each sample was centrifuged and frozen until analysis.

Analytical Procedures. Determination of urinary PGE2 was made by radioimmunoassay as described previously (28). Liquid chromatographic analyses were performed with a Bioanalytical Systems, Inc. (West Lafayette, IN) System 540 chromatographic system equipped with a 25-cm, 5-μm Biosep octadecylsulfone column and an LC-4B glassy carbon amperometric detector operated in series with a Bio-Instruments Model HS radiocative flow detector (Radiometric Instruments and Chemical Co., Inc., Tampa, FL) equipped with a 2.5-cm flow cell, as described previously (18) except where noted. The UV absorbance of the ANFT-MA peak was determined with a Model 155 variable wavelength detector (Beckman Instruments, Berkeley, CA). Frozen urine and bile samples were thawed and centrifuged at 2000 × g for 10 min prior to analysis for PGE2, ANFT, or ANFT-MA. For the determination of ANFT in urine, the sample was diluted 10- to 100-fold depending on ANFT content, and 20 μl were injected into the chromatograph with a mobile phase of methanol:0.1 M ammonium acetate, pH 6.0 (35:65), at a flow rate of 1 ml/min. ANFT was quantitated amperometrically at +750 mV versus silver-AgCl. For the determination of urinary ANFT-MA, 20 μl of undiluted urine were injected into the chromatograph with a mobile phase of methanol:0.1 M ammonium acetate, pH 6.0 (28:72), at a flow rate of 1 ml/min. ANFT-MA was quantitated amperometrically at +625 mV versus silver-AgCl. Quantitation of ANFT and ANFT-MA was made by measurement of peak height and comparison of this to the amperometric response of a chromatographically purified, radioiodinated standard injected immediately following each sample. The radiochemical purity of the standard was confirmed via the Flo-One detector, and the concentration was determined by liquid scintillation counting. Excretion values were calculated from urine volumes and are expressed as nmol/18 h. Urinary ANFT-MA was further characterized by chromatographically assisted hydrodynamic voltammetric analysis. A voltammogram of synthetic ANFT-MA and urinary ANFT-MA was obtained following repetitive injections of samples into the liquid chromatograph using an electrochemical detector poised at various potentials (29). Results are shown as peak height responses as a function of applied potential (Fig. 6). ϕ is a normalization factor by which the peak heights (i.e., current values) are expressed as a fraction of the value obtained at the largest potential utilized.

Statistical Analysis. Results are expressed as mean ± SE. Statistical analysis was performed with Student’s t test and analysis of variance of unpaired means, which gave equivalent expressions of significance.

RESULTS

Arachidonic acid mediated metabolism of [^14C]ANFT by ram seminal vesicle PHS is depicted in Fig. 1. Glutathione significantly altered metabolism. In the presence of 1 mM glutathione, binding of ANFT to the aqueous TCA precipitable fraction was not observed. In contrast, the amount of radioactivity in the TCA soluble fraction increased. Indomethacin completely inhibited arachidonic acid mediated metabolism. Subsequent studies were designed to determine whether the increase observed in the TCA soluble fraction with glutathione was due to thioether conjugate formation.

A HPLC chromatogram of the aqueous TCA soluble fraction is shown in Fig. 2. The peak eluting at 8.3 min showed chromatographic and electrochemical properties identical to those of the authentic ANFT-SG standard (Fig. 2, panel 1). This peak was not observed if indomethacin was present during the incubation (Fig. 2, Panel 2). Similar results were observed with radiochemical detection. The lack of product formation under conditions utilized in Fig. 2, panel 2 (ANFT plus glutathione) indicates the lack of a nonenzymatic reaction forming ANFT-
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Fig. 2. Effect of indomethacin on arachidonic acid mediated formation of ANFT-SG. Analysis was performed using HPLC with electrochemical detection. Conditions were the same as in Fig. 1 except 1 mM glutathione was present. Panel 1, absence of indomethacin; Panel 2, presence of 0.1 mM indomethacin. Peak A, ANFT-SG; Peak B, authentic ANFT.

SG. Substitution of either cysteine or N-acetylcysteine for glutathione during arachidonic acid mediated metabolism of ANFT by seminal vesicles resulted in the formation of a corresponding thioether conjugate with a distinct HPLC elution profile. The thioether conjugate formed in the presence of N-acetylcysteine has been identified as 2-amino-4-(5-nitro-2-furyl)-5-(N-acetylcystein-S-yl)thiazole (25). These results are consistent with ANFT metabolism by the hydroperoxidase activity of PHS and with glutathione reacting with activated ANFT to form a conjugate.

Effects of the peroxidase inhibitors PTU and MMI on ANFT-SG formation were examined using HPLC with electrochemical detection for quantification (Fig. 3). Arachidonic acid mediated increases in ANFT-SG were significantly reduced by PTU and MMI. Similar effects of PTU and MMI on ANFT metabolism were observed in the absence of glutathione. Arachidonic acid mediated binding to the TCA precipitable fraction was decreased from 0.94 to 0.1 to 0.11 ± 0.01 and 0.04 ± 0.01 nmol product with 1.3 mM PTU and MMI, respectively. In addition, total PHS hydroperoxidase metabolism of [14C]ANFT (1.3 nmol representing aqueous TCA soluble and precipitable fraction) produced following H2O2 addition to seminal vesicles was significantly inhibited with PTU. In contrast, indomethacin does not significantly alter peroxide mediated ANFT metabolism (30).

Alternative methods were assessed for the formation of ANFT conjugates. A reaction between PTU or MMI and ANFT was not indicated by either ANFT disappearance or product formation. PTU and MMI inhibition, like that of indomethacin illustrated in Fig. 2, were characterized by increased amounts of ANFT and the lack of additional products. Furthermore, PTU and MMI addition resulted in decreased formation of both TCA soluble and precipitable products of ANFT metabolism. In contrast, glutathione addition caused an increase in soluble products and a decrease in precipitable products (Fig. 1). The possible formation of ANFT-SG by glutathione S-transferases was assessed with liver cytosol and a commercial glutathione S-transferase preparation. These experiments failed to detect either a loss of ANFT or ANFT-SG formation. Thus, glutathione is reacting with PHS activated ANFT to form a conjugate.

Urine from rats given [14C]FANFT was examined for the presence of the mercapturic acid conjugate of activated ANFT (Fig. 4). The peak eluting at 45.5 min corresponded to the synthetic mercapturic acid conjugate. The peak collected at 45.5 min displayed UV visible absorption at 290 and 400 nm, characteristic of ANFT-MA (25). ANFT-MA represented approximately 3% of the total urinary radioactivity recovered in 2 h. Simultaneous monitoring with electrochemical detection confirmed the identity of this peak as being ANFT-MA. ANFT was the major urinary metabolite observed representing 33% of the recovered radioactivity. FANFT was not detected.

Disposition of ANFT-MA in rat was further assessed. Following administration of ANFT-SG to the isolated, perfused rat kidney, ANFT-MA is very rapidly and quantitatively excreted in the urine. A glomerular filtration rate of 0.70 ± 0.18 ml/min and a fractional excretion of sodium (3.7 ± 0.9%) were observed which compare favorably with the corresponding values for perfusion of FANFT and ANFT (17). ANFT-SG was completely removed from the perfusate within 20 min and appeared in the urine as ANFT-MA (97 ± 3% of recovered urinary ANFT products). Urinary ANFT-MA represented a 59 ± 8% recovery of administered ANFT-SG. Appreciable biliary excretion of radiolabeled metabolites was detected after i.v. infusion of 2-[14C]ANFT or 2-[14C]FANFT. However, neither ANFT-SG nor ANFT-MA was observed in bile. Because erythrocytes contain considerable amounts of glutathione and have been reported to possess weak peroxidative activity toward certain amine-containing substrates (31), ANFT was incubated

Fig. 3. Effects of PTU and MMI on arachidonic acid mediated formation of ANFT-SG. Conditions were the same as in Fig. 1 except 0.25 mM glutathione was present.

Fig. 4. Radiochromatogram of a 2-h urine sample from a rat given [14C]-FANFT. The solvent systems used to elute metabolites were: 0.1 M ammonium acetate buffer, pH 6.0, for 10 min; 10% methanol from 10 to 20 min; 10 to 55% methanol from 20 to 35 min; and 55% methanol from 35 to 55 min. Identification of metabolites was made by comparison with authentic standards.
with whole rat blood. However, neither ANFT-SG nor ANFT-MA was observed. Thus, although the specific site(s) of in vivo ANFT-MA production was not determined, ANFT-MA appears to qualify as a suitable marker for assessing peroxidase catalyzed metabolism of ANFT in vivo with urine rather than bile as a likely source.

A method was developed to assess urinary ANFT-MA using HPLC with electrochemical detection. A representative chromatogram which shows the amperometric detection of ANFT-MA is given in Fig. 5. An 18-h urine sample was obtained from a rat dosed with 150 mg/kg FANFT p.o. Urinary ANFT-MA was consistently absent from randomly selected control urine samples of rats not treated with FANFT. This is illustrated by the dashed line and arrow in Fig. 5 indicating the appearance of the base line in control urine samples. This chromatogram also indicates that no endogenous peaks are observed at the detector potential utilized. As a final confirmation of identity, the chromatographically resolved peak was found to display an electrochemical response identical to that of authentic ANFT-MA. This is documented in the hydrodynamic voltammogram illustrated in Fig. 6. The precision of the determination of ANFT-MA in a typical urine sample from a rat that excreted 9.2 nmol ANFT-MA/18 h was 5.22% relative SD (N = 5). Thus, a sensitive method was developed to allow the assessment of ANFT-MA as an index of in vivo peroxidase metabolism of ANFT.

The effects of PTU and MMI on ANFT-MA formation were investigated in vivo (Table 1). Because of the difficulty in obtaining sufficient [14C]FANFT for further in vivo experiments and the ability to quantitate ANFT-MA with electrochemical detection, the latter was used as the analytical tool for detecting ANFT-MA in this series of experiments. A p.o. dose of FANFT (150 mg/kg) was given so as to be approximately equivalent to the daily intake by rats consuming diet containing 0.2% FANFT in the standard tumor induction studies (13). Rats received equivalent molar doses of PTU and MMI 30 min prior to p.o. administration of FANFT. Urinary ANFT excretion was not altered by PTU or MMI. In contrast, ANFT-MA excretion was significantly reduced by PTU or MMI. Excretion of PGE2 was measured as an index of the catalytic potential of urinary tract PHS. PGE2 excretion was not altered by either PTU or MMI.

**DISCUSSION**

This is the first study to demonstrate that a product of PHS activated ANFT can be formed in vivo. This product was a thioether conjugate of ANFT previously identified as 2-amino-4-(5-nitro-2-furyl)-5-(N-acetylcystein-S-yl)thiazole (ANFT-MA). *In vitro* studies demonstrated that this conjugate is formed under conditions specific for PHS. In the presence of glutathione, only aqueous TCA soluble products of arachidonic acid mediated ANFT metabolism were observed. TCA precipitable (protein bound) products of metabolism were not observed. This change from TCA precipitable to soluble metabolism was shown to be due to formation of ANFT-SG. Indomethacin, like aspirin (14), is an inhibitor of the fatty acid cyclooxygenase.
activity of PHS and inhibited total arachidonic acid mediated metabolism (TCA soluble, TCA precipitable, and ANFT-SG). PTU and MMI also inhibit total arachidonic acid mediated metabolism of ANFT. However, PTU and MMI also prevented peroxide initiated metabolism while indomethacin did not (9, 10). PTU and MMI are both substrates for a variety of peroxidases including the hydroperoxidase activity of PHS (32). Thus, PTU and MMI probably inhibit ANFT metabolism by functioning as alternative substrates (9). Because indomethacin inhibits the fatty acid cyclooxygenase and not prostaglandin hydroperoxidase activity of PHS, indomethacin inhibits arachidonic acid but not peroxide mediated ANFT metabolism.

Thus, inhibition by PTU and MMI is consistent with ANFT metabolism by the hydroperoxidase activity of PHS (9). PHS is the only peroxidase present in rat seminal vesicles (33) and is also the only peroxidase tested which metabolizes ANFT (22). Other mechanisms for formation of thioether conjugates of ANFT such as a direct reaction with PTU and MMI or a glutathione S-transferase mediated reaction were not detected. In addition, previous experiments have demonstrated that NADPH supplemented liver microsomes do not oxidize ANFT aerobically (21). Thus, glutathione derived conjugates of PHS activated ANFT appear to be a good index of PHS activity.

Metabolism and disposition studies have revealed some unusual characteristics of the FANFT/ANFT model. Renal deconjugation of FANFT and subsequent excretion of ANFT are responsible for more ANFT excreted in the urine when FANFT is administered to rats rather than equimolar amounts of ANFT (17, 18, 20). This metabolic/excretory coupling explains the paradox that FANFT is a more potent urothelial carcinogen when fed to rats than is ANFT, although ANFT is thought to be the proximate carcinogen (13, 34, 35). Thus, with respect to the pathogenesis of urothelial malignancies induced by FANFT, it is important to examine the fate of ANFT generated with the administration of FANFT.

ANFT and its thioether metabolites were examined in vivo following [14C]FANFT administration. No FANFT is recovered in urine from these rats. ANFT represented the major metabolite in urine. ANFT-MA was also observed. ANFT-MA was not observed in bile from FANFT treated rats or in urine from controls (non-FANFT treated rats). The lack of ANFT-MA or ANFT-SG in bile from rats fed FANFT suggests that these conjugates are not produced in the liver (36). Furthermore, whole blood does not appear to be the site of conjugate formation. The site of ANFT-MA formation was not determined. Because thiomethyl and mercapturic acid conjugates of many foreign compounds are known to be urinary metabolites of previously formed glutathione conjugates (37, 38), the fate of ANFT-SG was assessed in the isolated perfused kidney. ANFT-SG was rapidly converted to ANFT-MA by the kidney and excreted in urine. Thus, endogenous glutathione can serve as a trap for PHS activated ANFT, and urinary ANFT-MA may be derived from ANFT-SG in the systemic circulation or urinary tract.

Prostaglandins have been postulated to be involved in several steps in the carcinogenic process in addition to many important physiological processes (39, 40). PHS activity is present at high levels in tissues of the urinary tract (41) and has been reported in transitional epithelium of rats (15), rabbits (6, 28), dogs (42), and humans (43). Administration of an inhibitor of the fatty acid cyclooxygenase activity of PHS would alter a multiplicity of processes involving prostaglandins. In a recent study assessing the effects of aspirin on the two-stage model of FANFT bladder carcinogenesis, aspirin was found to affect both the initiation and promotion stages (44). A more selective inhibitor than aspirin would be a useful tool to further investigate the involvement of PHS in FANFT-induced bladder cancer. PTU and MMI are more selective inhibitors of carcinogen activation by PHS (9) and were chosen for this study. In addition to being peroxidase inhibitors, both drugs have been extensively used clinically (45). Each is capable of reducing cooxidation of amines by PHS without affecting the production of PGE2 (9, 46). PTU and MMI are well tolerated at relatively high acute p.o. doses (47). PTU has been shown to be excreted primarily unchanged in rat urine (48), suggesting exposure of the urinary tract tissue to high levels of this inhibitor. Prior administration of either PTU or MMI was found to have a specific inhibitory effect on ANFT-MA excretion (Table 1). In contrast, neither PTU nor MMI altered ANFT or PGE2 excretion. Urinary PGE2 levels are thought to reflect urinary tract PHS catalytic activity (49). These in vivo effects of PTU and MMI are completely consistent with their in vitro effects on ANFT activation. These results further emphasize the proposed peroxidatic nature of ANFT metabolism. While processes other than peroxidative cannot be excluded for the formation of ANFT-MA, it is unclear how both PTU and MMI could exert the same inhibitory effect upon the same nonperoxidative metabolic process. The involvement of additional peroxidases in the in vivo metabolic formation of ANFT-MA also cannot be excluded but may be considered unlikely, in view of the failure to demonstrate metabolism of ANFT by several peroxidases other than PHS. 

There are other potential enzymatic mechanisms for FANFT/ANFT metabolism. As illustrated in Fig. 4, there are many radioactive peaks observed following HPLC analysis of urine from rats administered [14C]FANFT. Our studies focused entirely on the formation of ANFT-MA. ANFT-MA represents approximately 5% of the ANFT excreted by Fischer rats during 18 h following administration of FANFT and less than 0.5% of the dose of FANFT. Nitroreduction of ANFT or FANFT would be expected to occur. Each of their nitrile products of reduction has been identified (21, 50). The nitrite product of ANFT was observed in urine from mice given FANFT (51) and during incubation of ANFT with rat urothelial cells (52). A thioether conjugate of a reduction product may also be formed. However, such a product has never been identified and would have chemical properties entirely different from those of the ANFT-MA product of PHS metabolism. For example, ANFT-MA has an absorbance maximum in the range of 380–410 nm which is consistent with an intact 5-nitrofuran ring (25). This absorbance maximum is absent in nitroreduction products of FANFT and ANFT (21, 50).

A model has been developed, based on in vitro results, depicting initiation of carcinogenesis by PHS (9). According to this model, four sites have been identified at which PHS activation of aromatic and heterocyclic amines to bind DNA can be prevented. They are: site 1, synthesis of peroxide substrate (indomethacin and aspirin inhibition); site 2, activation of carcinogen (PTU and MMI inhibition); site 3, conversion of activated carcinogen back to parent compound (ascorbic acid inhibition); and site 4, inactivation of activated carcinogen (glutathione conjugation). Previous studies have demonstrated only site 1 inhibition in vivo (15, 16, 44). In the present study, the discovery of urinary ANFT-MA and the inhibition of its synthesis by PTU and MMI demonstrates that both sites 2 and 4 can also occur in vivo. This model is useful for understanding the initiation of carcinogenesis by aromatic and heterocyclic amines. Peroxidase inhibitors may be attractive choices as
agents for pharmacologically altering the initiation of FANFT-induced lesions and tumors in cofeeding studies.

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