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

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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-\(\alpha\)-propyl-2-thiouracil and methimazole, significantly reduced ANFT binding to chloroacetic acid precipitable material and glutathione conjugate formation. Isolated perfused kidneys rapidly converted the glutathione conjugate to its corresponding mercapturic acid (ANFT-MA). With both biochemical and electrophoresis techniques, ANFT-MA was identified in the urine of rats given \(N\)\(\beta\)-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide, the carcinogenic \(N\)-(formyl analogue of ANFT. ANFT-MA was the major urinary metabolite with \(N\)\(\beta\)-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide not detected. A 30-min pretreatment with 6-\(\alpha\)-propyl-2-thiouracil and methimazole significantly reduced urinary excretion of ANFT-MA in rats given \(N\)\(\beta\)-[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 nmol/18 h, respectively. Peroxidase inhibitor pretreatment did not alter the excretion of ANFT or prostaglandin E\(_2\). 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 \(P\)\(H\)S bind DNA (5-7). \(P\)HS consists of two distinct enzymatic activities, \(P\)HS cyclooxygenase and prostaglandin hydroperoxidase (8). \(P\)HS cyclooxygenase catalyzes the biodegradation of arachidonic acid to the cyclic endoperoxide PGG\(_2\). Prostaglandin hydroperoxidase is responsible for the reduction of PGG\(_2\). During this reduction, prostaglandin hydroperoxidase will oxidize suitable co-substrates, i.e., aromatic and heterocyclic amines (9, 10). In this manner, the hydroperoxidase activity of \(P\)HS activates certain amines to electrophiles which bind DNA. Thus, this suggests a potential role for \(P\)HS in the genesis of malignancies by certain carcinogenic amines. However, only a few in vivo investigations have supported this hypothesis.

\(P\)HS catalyzed binding of 2-naphthylamine to DNA in vitro results in the formation of adducts derived from 2-amino-1-
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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 peaks observed in the presence of indomethacin are near the limit of detection. To examine formation of ANFT-SG by erythrocytes, whole rat blood was collected by cardiac puncture and treated with heparin to prevent clotting, and 3 individual 1-ml aliquots were incubated with 200 μM ANFT in air with shaking for 1 h at 37°C. The protein was precipitated with 10% TCA and 25-μl aliquots were analyzed by liquid chromatography (25) for ANFT-SG. To assess glutathione S-transferase activity, rat liver cytosol was prepared by centrifugation of rat liver homogenate (26) at 102,000 × g and was incubated with either 1.0 mM ANFT or 1.0 mM unlabeled FAN FT 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-MANF 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 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, NY) 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 [3H]FAN FT plus 10 μmol of unlabeled FAN FT 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-MANF and/or ANFT-SG by liquid chromatography as described above.

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, NY) 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 [3H]FAN FT plus 10 μmol of unlabeled FAN FT 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-MANF and/or ANFT-SG by liquid chromatography as described above.

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 these conditions utilized in Fig. 2, panel 2 (ANFT plus glutathione) indicates the lack of a nonenzymatic reaction forming ANFT-

RESULTS

Arachidonic acid mediated metabolism of [14C]FAN FT 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-

Arachidonic acid mediated metabolism of ANFT. Standard incubation as described in "Materials and Methods" was used. Binding of ANFT to protein was assessed following ethyl acetate extraction and subsequent precipitation of protein with TCA. GST, glutathione; INDO, indomethacin.

Fig. 1. Arachidonic acid mediated metabolism of ANFT. Standard incubation as described in "Materials and Methods" was used. Binding of ANFT to protein was assessed following ethyl acetate extraction and subsequent precipitation of protein with TCA. GST, glutathione; INDO, indomethacin.
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-transferase 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 with 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.

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![Figure 2](image1.png)

**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.

![Figure 3](image2.png)

**Fig. 3.** Effects of PTU and MMI 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 0.25 mM glutathione was present.

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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-furanyl)-5-(N-acetylcysteine-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-transferase 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.

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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.

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**Table 1** Effect of propylthiouracil and methimazole on urinary metabolites from rats dosed with FANFT

<table>
<thead>
<tr>
<th>Condition</th>
<th>PGE2 (ng/18 h)*</th>
<th>ANFT (nmol/18 h)</th>
<th>ANFT-MA (nmol/18 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (5)</td>
<td>174 ± 34</td>
<td>307 ± 47</td>
<td>14.8 ± 2.1</td>
</tr>
<tr>
<td>+ 250 mg/kg PTU (5)</td>
<td>178 ± 28</td>
<td>293 ± 82</td>
<td>7.9 ± 0.8†</td>
</tr>
<tr>
<td>+ 167.5 mg/kg MMI (4)</td>
<td>150 ± 43</td>
<td>278 ± 43</td>
<td>6.2 ± 1.1†</td>
</tr>
</tbody>
</table>

* Rats were pretreated p.o. with dimethyl sulfoxide or inhibitor 30 min prior to p.o. dosing with 150 mg/kg FANFT.
† Numbers in parentheses, number of rats per group.
‡ Significantly different from control at P < 0.02.
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 ram 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 de- formylation 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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