Biotransformation of the Bladder Carcinogen N-[4-(5-Nitro-2-furyl)-2-thiazolyl]formamide in Mice

Santhanam Swaminathan and George T. Bryan

Division of Clinical Oncology, Department of Human Oncology, Wisconsin Clinical Cancer Center, Madison, Wisconsin 53792

ABSTRACT

The biotransformation of N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (FANFT), a potent urinary bladder carcinogen, was studied in mice. About 82% of radioactivity was excreted as $^{14}$CO$_2$ within 36 hr after intragastric administration of N-[4-(5-nitro-2-furyl)-2-thiazolyl]-[1$^{14}$C]formamide, suggesting its deformylation to 2-amino-4-(5-nitro-2-furyl)thiazole (ANFT). The latter was formed in vitro as a product following incubation of FANFT with mouse liver homogenates. Chromatographic analysis of mouse urine obtained 24 hr after the i.p. administration of N-[4-(5-nitro-2-furyl)-2-[1$^{14}$C]thiazolyl]formamide revealed excretion of ANFT and a deaminated metabolite. The latter was characterized chromatographically and spectral properties similar to those exhibited by a compound derived from the in vitro nitrification of ANFT. This metabolite was isolated from urine of FANFT-fed animals and from in vitro enzymatic reduction of ANFT with mouse liver homogenates. The isolated products had chromatographic and spectral properties and a mass spectral fragmentation pattern similar to that of a compound obtained by catalytic reduction of ANFT with palladium and activated carbon. Spectroscopic analyses established the structural identity of the chemical reduction product as 1-[N-(2-aminothiazolyl)]-3-cyano-1-propanone (ATCP). Since the chromatographic properties of the enzymatically derived compound and the urinary metabolite were identical to those of a compound obtained by chemical reduction, they must be structurally the same and thus correspond to ATCP. About 5% of the urinary metabolites of FANFT is ATCP, and thus ATCP is quantitatively a minor excretory product. ATCP was far less active than was ANFT or FANFT in the Ames mutagenicity assay with Salmonella typhimurium TA 100.

INTRODUCTION

Numerous 5-nitrofurathiiazole analogues and related chemicals demonstrated oncogenic activity for a variety of species and tissue sites (reviewed in Ref. 1). Among these chemicals, FANFT is a versatile and one of the most potent experimental bladder carcinogens, as it has been shown to induce carcinoma of the bladder in dogs (5), rats (8), mice (3), and hamsters (4). Chronic administration of FANFT p.o. to Swiss mice resulted in a high incidence of urinary bladder carcinoma and a lower incidence of leukemia and forestomach tumors (3, 6). The molecular bases for metabolic activation of and relative tissue susceptibilities to carcinogenic 5-nitrofurathiiazoles are not known. Nitroreduction involving nitroso- and N-hydroxylamino intermediates (20) and cooxidation by prostaglandin endoperoxide synthetase (13) have been proposed. Earlier metabolic studies showed that FANFT was converted to ANFT in vitro (12, 25), and the latter was detected in the urine of rats and mice following administration of FANFT (27). Thus, it was proposed that ANFT might be a proximate carcinogen in FANFT-induced bladder carcinogenesis in these animals (3). However, when ANFT was tested for carcinogenicity, it produced bladder tumors in rats (28), but not in mice (3). Instead, ANFT caused predominantly forestomach squamous cell tumors in mice (3). Similar differences in tissue susceptibilities were observed between mice and rats with other related analogues such as N-[4-(5-nitro-2-furyl)-2-thiazolyl]acetamide, formic acid 2-[4-(5-nitro-2-furyl)-2-thiazolyl]hydrazide, and related analogues (reviewed in Ref. 1). To examine the metabolic factors associated with the above observed differences in tissue susceptibilities, we investigated the metabolism of FANFT and ANFT in mice. Since the carcinogenicity and mutagenicity of the 5-nitrofurans have been linked to reductive metabolism (reviewed in Ref. 20), we studied the reduction products formed from FANFT. We previously reported data obtained from an in vitro (21) and in vivo (19) metabolic study of NFT, and we identified a saturated ketonitrile TCP as a nitroreduction product. Recently, an unsaturated ketonitrile was reported to be tentatively identified as a reduction product of FANFT in vitro, using rabbit kidney microsomes (29). We report here the metabolism of FANFT, involving deformylation and nitroreduction reactions, to yield the saturated ketonitrile ATCP.

MATERIALS AND METHODS

Materials. FANFT, ANFT, and 2-bromoacetyl-5-nitrofuran were purchased from Saber Laboratories, Inc., Morton Grove, IL. They were recrystallized prior to use, and the crystalline preparations were >99% pure as evaluated by chromatographic methods. Palladium (5%) on activated carbon, thiourea, deuterchloroform, and TMS were obtained from Aldrich Chemical Co., Inc., Milwaukee, WI. Ethyl acetate, acetonitrile, and methanol were bought from Burdick and Jackson Laboratories, Inc., Muskegon, MI. Sodium $^{14}$C]formate (approximately 50 mCi/mmol) and $^{14}$C]thiourea (approximately 50 mCi/mmol) were purchased from Amersham Corp., Arlington Heights, IL. Female Swiss mice weighing about 25 g were purchased from Harlan-Sprague-Dawley, Inc., Madison, WI.

Syntheses of Radioactive Compounds. $^{2-14}$C]ANFT was synthesized from recrystallized 2-bromoacetyl-5-nitrofuran and $^{14}$C]thiourea as...
S. Swaminathan and G. T. Bryan
described (22). The final product (330 /µCi/mmol) was recrystallized in
ethanol, and the crystalline preparations were >99% pure as evaluated
by radiochromatographic scans and GC.

[2-14C]FANFT was prepared by dissolving 1 mmol of [2-14C]ANFT in
0.5 ml of pyridine and 2 ml of concentrated formic acid. The solution was
warmed to 70° for 3 hr, and [2-14C]FANFT (Chart 1) precipitated out by
dilution with 10 ml of ice-cold distilled water. The precipitate was collected
by filtration, rinsed with 50 ml of distilled water and 25 ml of ice-cold
methanol, and air dried. Recrystallization from aqueous dimethyl sulfoxi-
dide yielded [2-14C]FANFT (30% yield, 190 /µCi/mmol). It was found to be
>99% radiochemically pure.

(formyl-14C)FANFT was synthesized using ANFT (1 mmol) in 1.0 ml of
dimethyl sulfoxide and 0.5 ml of concentrated formic acid containing 1.0
mCi of sodium [14C]formate. The mixture was refluxed in a small round-
bottomed flask for 2 hr, cooled, and diluted with 10 ml of ice-cold distilled
water. [formyl-14C]FANFT (Chart 1) was isolated as described above.
The final product (32% yield, 134 /µCi/mmol) was identical to authentic
FANFT and was determined to be chromatographically and radiochemi-
cally pure.

Chemical Reduction of ANFT. ANFT was reduced by a method
described earlier (21). The reaction mixture was filtered and extracted
with diethyl ether. The material was concentrated and purified on a
column of silica gel using ethyl acetate. The sample was recrystallized in
methanol. The crystalline preparations were found to be >99% pure by
HPLC methods. The purified sample was pale yellow (m.p. 188–192°,
with decomposition). The compound was very soluble in water, methanol,
and other polar organic solvents. It was stable at 25° and could be
stored for months at −20°.

Distribution and Excretion of [2-14C]FANFT and [formyl-14C]FANFT.
Four female Swiss mice weighing about 25 g were given [2-14C]FANFT
(1.6 µCi, 8.6 nmol) or [formyl-14C]FANFT (1.1 µCi, 8.3 nmol) in 0.5 ml of
polyethylene glycol by stomach tube. They were immediately housed
individually in glass metabolism cages for the trapping of expired CO2 in
glass towers containing ethanolamine:methanol (1:4). Radioactivity was
gas analyzed by a liquid scintillation procedure described previously (2,19).

Analyses of Urinary Metabolites. Urine samples were collected from
the mice for 36 hr in a flask maintained in an ice-bath after p.o. admin-
istration of [2-14C]FANFT, as described above. In experiments involving
i.p. administration, 6 female Swiss mice were given [2-14C]FANFT (1.0
µCi, 4.2 µmol) in 0.2 ml of 25% dimethyl sulfoxide. Urine samples were
collected for 24 hr at 12-hr intervals, filtered, and diluted to a 25-ml
volume with distilled water. An aliquot of the urine was extracted 5 times
with an equal volume of ethyl acetate or diethyl ether. The extracts were
pooled, and the solvent was removed by distillation in a vacuum. The residue
was dissolved in a small volume of methanol, and appropriately
diluted samples were subjected to chromatographic analysis.

Isolation of ATCP from Urine. Twenty female Swiss mice were placed
in 4 metabolism cages and were fed ground Wayne Lab-Blox containing
0.2% FANFT for 48 hr. Urine (70 ml) collected in glass containers
maintained in dry ice was pooled and filtered. The filtrate was extracted
5 times with equal volumes of ethyl acetate. The organic extracts were
pooled and removed by distillation in a vacuum. The residue was dis-
solved in 2.0 ml of methanol and subjected to chromatography on a
column of silica gel using ethyl acetate as the eluting solvent. The eluant
fraction corresponding to that of ATCP was pooled, evaporated to dryness,
and then purified by preparative TLC on silica gel plates using an
ethyl acetate:n-hexane (3:2, v/v) mixture as the developing solvent.
The purified metabolite was then subjected to UV, HPLC, TLC, and mass
spectral analyses.

Assay for Deformylation and Nitroreduction. Mice, weighing about
25 to 30 g, were decapitated. The livers were immediately removed,
perfused, rinsed with ice-cold 1.15% potassium chloride, and then ho-
moxygenized in a Potter-Elvehjem homogenizer using 10 ml of potassium
chloride solution/g, wet weight, of liver. The homogenate was dialyzed
overnight at 4° against 100 volumes of 0.1 M Tris buffer, pH 7.2, and
used for the deformation assays.

The assay mixture in a 2-ml volume contained 0.5 µmol [2-14C]FANFT
(0.08 /µCi in 0.2 ml dimethylformamide), 0.18 µmol Tris buffer, pH 7.2,
and 1.0 ml of mouse liver homogenate. The incubation was conducted at
37° for a specified period of time. The reaction was stopped by adding
0.5 ml of 5% zinc sulfate solution and 0.5 ml of dimethylformamide
and cooled at 4° for 30 min. The sample was centrifuged at 10,000 × g for
15 min, and the supernatant fraction was extracted 3 times with buffer-
saturated diethyl ether (peroxide free). The ether layer was pooled,
evaporated to dryness under vacuum, and then dissolved in a small
volume of methanol prior to streaking on TLC plates.

The nitroreduction assay was conducted by a similar procedure pub-
lished earlier (21), using 10,000 × g mouse liver homogenate prepara-
tions. At the end of incubation, the mixture was boiled for 15 min and
centrifuged at 15,000 × g for 30 min. The supernatant fraction was
filtered and extracted 5 times with equal volumes of diethyl ether. The
ether extract was evaporated to dryness and then subjected to chro-
matography on a column (1.2 × 52.5 cm) of silica gel. The column was
eluted with ethyl acetate, at a flow rate of 39 ml/hr, and the samples
collected between 40 and 65 ml of elution volume were pooled, evapo-
rated to dryness, and then subjected to HPLC and mass spectral
analyses.

Chromatography and Spectroscopy. HPLC was performed on a
Micromeritics 7000B equipped with Model 730 universal injection valve
and interfaced with a Varian Varichrom variable wavelength detector.
The instrument was fitted with a 25-cm Whatman Partisil PXS 10/25
ODS column. The HPLC was operated isocratically under constant-flow
mode using an acetonitrile:water (1:1, v/v) mixture as the eluting solvent.

TLC was performed on 0.1-mm No. 13181 silica gel plates (Eastman
Kodak Co., Rochester, NY) or on cellulose plates (E. Merck Laboratories,
Inc., Elmsford, NY). The solvent systems used with silica gel plates and
Rs determined for ATCP, ANFT, and FANFT, respectively, are: ethyl
acetate:n-hexane (2:3), 0.20, 0.32, and 0.41; and benzene:chloro-
form:methanol (10:8:1), 0.50, 0.69, and 0.80. Cellulose plates were
developed with aqueous solvent systems of 2% methanol or 2% formic
acid. The Rs obtained for ATCP, ANFT, and FANFT, respectively, are:
2% methanol, 0.60, 0.12, and 0.02; and 2% formic acid, 0.8, 0.22, and
0.06. Radioactivity on TLC was monitored in certain instances using a
Packard 7201 radiochromatogram scanner equipped with a disc integra-
tor. Alternatively, the chromatograms were cut into small strips and
counted in a Tracer Analytic Mark III Model 6882 liquid scintillation
counter, after adding 10 ml of organic counting solvent (Amersham
Corp., Arlington Heights, IL).

GC was performed in a Hewlett Packard Model 5710A chromatograph
equipped with an electron capture detector. Samples were injected onto a
2-mm (inside diameter), 6-foot-long glass column containing 3% OV-1
on 80/100 Chromosorb W. Argon:methane (95.5, v/v) was used as the
carrier gas at a flow rate of 20 ml/min. The chromatograms were run
isothermally at 210°.

Chart 1. Structure of FANFT. * position of 14C label.

2332 C A N C E R R E S E A R C H V O L . 4 4

Downloaded from cancerres.aacrjournals.org on April 14, 2017. © 1984 American Association for Cancer Research.
testinal tract and became readily available to metabolic and the formyl group was lost as 14CO2. Deformylation appeared to than the levels observed in mice receiving [2-14C]FANFT, since of about 5 tons. UV absorption spectra were taken in a Beckman 25 ANFT or its further metabolites, are the predominant constituents suggest that the deformylated products of FANFT, possibly correlate with major target tissues susceptible to FANFT carcin-
activity (Table 1). The tissue distribution of radioactivity did not was present in the urine after 36 hr. Distribution of radioactivity was excreted in urine, feces, and CO2 36 hr after i.g. administration of [2-14C]FANFT and [formyl-14C]FANFT, respectively. The urinary system was the major route for the excretion of [2-14C]FANFT, as 42% of recovered radioactivity was present in the urine after 36 hr. Distribution of radioactivity in tissues revealed liver and stomach displaying the highest mean activity (Table 1). The tissue distribution of radioactivity did not correlate with major target tissues susceptible to FANFT carcin-
genicity. The urine, feces, and tissue levels of radioactivity in mice administered [formyl-14C]FANFT were significantly lower than the levels observed in mice receiving [2-14C]FANFT, since the formyl group was lost as 14CO2. Deformylation appeared to be a rapid and highly significant route in view of the large production of 14CO2 (82%) following administration of [formyl-
14C]FANFT (Chart 2). In contrast, production of 14CO2 was neg-
ligible in mice given [2-14C]FANFT (Chart 2). These data strongly suggest that the deformylated products of FANFT, possibly ANFT or its further metabolites, are the predominant constituents present in urine and that the tissues are probably exposed to and incorporate primarily these products. Confirmation of the deformylation of FANFT to ANFT and its further biotransformation comes from in vitro studies. Deformylation of FANFT to ANFT. Chart 3 shows the profile of the radioactivity of the TLC following incubation of [2-14C] FANFT with mouse liver 10,000 x g supernatant preparations. In the absence of a 10,000 x g supernatant fraction in the incubation mixture, only one radioactive peak was detected near the origin, and it corresponded with FANFT. No radioactivity at the region corresponding with ANFT (Chart 3, Curve A) was detected. In contrast, incubation of [2-14C]FANFT with liver 10,000 x g supernatant preparations resulted in an additional radioactive peak (Chart 3, Curves B and C) whose relative mobility corresponded with synthetic ANFT. The amount of ANFT formed increased with increasing duration of incubation along with a concomitant decrease in the level of FANFT. In separate studies, urine specimens were collected during the first and the subsequent 12 hr following i.p. administration of [2-
14C]FANFT. About 20% of the administered radioactivity was excreted during the first and the subsequent 12-hr periods, respectively. About 17% of the radioactivity from the 12-hr urine sample could be extracted with diethyl ether. Analysis of thin-layer chromatograms (cellulose, 2% formic acid system) of the ether extract revealed a major band of radioactivity correspond-
ing with ANFT (Rf = 0.25). The presence of the ANFT band was more pronounced in urine collected during the first 12 hr (data not shown). This was further confirmed by GC analysis. The GC of synthetic ANFT (Chart 4A) showed a peak at a retention time of about 6.5 min. A peak with a similar retention time could be observed in the urine extract after 12 and 24 hr of administration of FANFT (Chart 4, B and C). On the contrary, the urine extract from control animals did not show any peak at a retention time

RESULTS

Distribution and Excretion of [2-14C]FANFT and [formyl-
14C]FANFT. The distribution of radioactivity 36 hr after i.g. administration of 14C-labeled compounds to mice is presented in Table 1. FANFT was absorbed from the lumen of the gastroin-
testinal tract and became readily available to metabolic and excretory processes. A total of about 84 and 93% of recovered radioactivity was excreted in urine, feces, and CO2 36 hr after i.g. administration of [2-14C]FANFT and [formyl-14C]FANFT, respectively. The urinary system was the major route for the excretion of [2-14C]FANFT, as 42% of recovered radioactivity was present in the urine after 36 hr. Distribution of radioactivity in tissues revealed liver and stomach displaying the highest mean activity (Table 1). The tissue distribution of radioactivity did not correlate with major target tissues susceptible to FANFT carcin-
genicity. The urine, feces, and tissue levels of radioactivity in mice administered [formyl-14C]FANFT were significantly lower than the levels observed in mice receiving [2-14C]FANFT, since the formyl group was lost as 14CO2. Deformylation appeared to be a rapid and highly significant route in view of the large production of 14CO2 (82%) following administration of [formyl-
14C]FANFT (Chart 2). In contrast, production of 14CO2 was neg-
ligible in mice given [2-14C]FANFT (Chart 2). These data strongly suggest that the deformylated products of FANFT, possibly ANFT or its further metabolites, are the predominant constituents

<table>
<thead>
<tr>
<th>Tissue or specimen</th>
<th>[2-14C]FANFT</th>
<th>[formyl-14C]FANFT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine</td>
<td>42.2 ± 5.9</td>
<td>8.6 ± 0.8</td>
</tr>
<tr>
<td>Feces</td>
<td>38.8 ± 5.7</td>
<td>3.0 ± 0.8</td>
</tr>
<tr>
<td>CO2</td>
<td>3.0 ± 0.2</td>
<td>61.7 ± 5.7</td>
</tr>
<tr>
<td>Liver</td>
<td>3.0 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Stomach</td>
<td>2.6 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>Intestines</td>
<td>2.5 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>0.8 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>0.5 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>0.2 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>0.1 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Bladder</td>
<td>0.1 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>Carcass</td>
<td>6.4 ± 2.0</td>
<td></td>
</tr>
</tbody>
</table>

*Three mice were given [2-14C]FANFT (1.5 μCi, 344 μmol/kg) by stomach tube. The results are the percentage of the recovered 14C excreted in 36 hr. Total recovery of radioactivity was 83.3% of the administered dose.

*Four mice were given [formyl-14C]FANFT (1.1 μCi, 344 μmol/kg) by stomach tube. The results are the percentage of administered radioactivity after 36 hr. Quantitations in tissues were not made because of the presence of 14C at very low levels.

*Mean ± S.D.

JUNE 1984

Biotransformation of FANFT in Mice

Chart 2. Production of 14CO2 by mice following administration of [2-14C]FANFT (open circle) and [formyl-14C]FANFT (closed circle). Four female Swiss mice were given [2-14C]FANFT (1.5 μCi, 8.8 μmol) or [formyl-14C]FANFT (1.1 μCi, 8.3 μmol) by stomach tube. Expired CO2 was trapped as described in "Materials and Methods," and the radioactivity was measured. Points, mean; bars, S.D.
S. Swaminathan and G. T. Bryan

FANFT ANFT

Radioactivity

Retention Time (min)

Chart 3. Radioscanning of the TLC after incubation of [2-14C]FANFT with mouse liver homogenate. The samples were prepared as described in "Materials and Methods," chromatographed on cellulose plates, developed with 2% formic acid in water, and scanned on a radiochromatogram scanner. Curve A, control, containing no 10,000 × g supernatant fraction in the incubation mixture; Curve B, after incubation with 10,000 × g supernatant fraction for 15 min; Curve C, after incubation with 10,000 × g supernatant fraction for 60 min; Curve D, synthetic standard mixture of [2-14C]ANFT and [2-14C]FANFT.

Nitroreduction of FANFT and ANFT. TLC (cellulose, 2% methanol system) of urine obtained 12 hr after i.p. administration of [2-14C]FANFT revealed 2 major spots (Rfs, 0.12 and 0.75) and 2 minor spots (Rfs, 0.30 and 0.60) (Chart 5). The metabolite corresponding with an Rf of 0.12 was confirmed to be ANFT, the deformylated product of FANFT. Since the prevalence of nitroreduction has been well documented earlier with closely related 5-nitrofuranylthiazole analogues (12, 20, 23, 24) to examine whether any of the urinary metabolites would correspond with the products resulting from nitroreduction, [2-14C]FANFT was incubated anaerobically in vitro, under reductive conditions described earlier (21), using a mouse liver 10,000 × g supernatant fraction and NADPH. After incubation at 37°, the samples were maintained in a boiling water bath for 15 min and centrifuged at 10,000 × g. The aqueous solution was chromatographed on plastic cellulose plates, as described above, and about 1-cm strips were cut and counted in a liquid scintillation counter. Radioactivity was detected at 4 regions with Rf values of 0.12, 0.46, 0.60, and 0.75 (Chart 5). Incubation of [2-14C]ANFT under similar conditions of reduction and analysis by TLC also showed radioactivity Rf of 0.12, 0.30, 0.60, and 0.75 (Chart 5). Formation of similar products from both [2-14C]FANFT and [2-14C]ANFT in vitro could be due to the deformylation of FANFT to ANFT under the conditions of reaction. Since the products corresponding with Rf 0.30, 0.60, and 0.75 were generated in vitro under conditions conducive for nitroreduction, and as they were observed also in the urine of animals given [2-14C]FANFT, it is suggested that FANFT is deformylated to ANFT (Rf, 0.12) and reduced to generate the metabolites with Rf values, 0.30, 0.60, and 0.75. The structural identity of the metabolite corresponding

Chart 4. GC elution traces of synthetic ANFT (8 ng) (A) and diethyl ether extract of 5 ml urine collected during 12 hr (1 µl in 1.5 ml) (B) and 24 hr (1 µl in 0.3 ml) (C) in FANFT-treated, and control animals (1 µl in 1.25 ml) (D).

Chart 5. Radioactivity in cellulose thin-layer chromatograms developed with 2% methanol in water. ——, diethyl ether extract of urine collected following i.g. administration of [2-14C]FANFT, 10 µl in 1.0 ml; ——, filtrate of urine collected during 12 hr after i.p. administration of [2-14C]FANFT, 20 µl; ——, reaction mixture following incubation of [2-14C]FANFT with 10,000 × g supernatant fraction for 1 hr in vitro, approximately 10 µl; ——, reaction mixture following incubation of [2-14C]-ANFT with 10,000 × g supernatant fraction for 1 hr in vitro, approximately 10 µl.
to the Rf of 0.60 has been studied and reported below, while the chemical identity of others remains to be investigated.

**Chemical Characteristics of ATCP.** A UV absorption spectrum of synthetic ATCP compound in methanol showed maxima at 222 nm (ε = 15.5 mm⁻¹ cm⁻¹) and at 305 nm (ε = 3.8 mm⁻¹ cm⁻¹). The IR spectrum (KBr pellet) had characteristic bands at 3400, 2270, and 1690 cm⁻¹ corresponding with -NH₂, -CN, and C=O, respectively (Chart 6). The proton nuclear magnetic resonance spectrum shown in Chart 7 revealed a chemical shift at 7.5 ppm corresponding with the single proton at position 5 of the thiazole ring. This showed the intactness of the thiazole ring during the reduction steps. A broad singlet at 5.0 ppm was observed and is characteristic of the protons of the amino group. In addition, 2 triplets each corresponding to 2 protons were seen at δ = 2.7 and 3.3 ppm, suggesting the presence of 2 methylene protons being adjacent to one another.

A low-resolution mass spectrum showed the molecular ion at m/e 181 (C₇H₇N₃OS) with additional fragment ions at m/e 141, 127, 100, and 99. The relative abundance of the ions observed along with a possible fragmentation pattern is shown in Chart 8A. The fragmentation pattern observed here is very similar to that reported earlier by us (21) for a closely related ketonitrile, TCP. These spectroscopic data are consistent with the structure of ATCP; thus, the chemical identity of ATCP was established.

**Formation of ATCP from ANFT and FANFT in Vitro.** TLC of synthetic ATCP performed on a cellulose system described earlier exhibited Rf 0.60. TLC of the ether extract obtained after incubation of FANFT or ANFT with mouse liver cytosol (5); and the metabolite isolated from urine of FANFT-fed animals (C).

On HPLC analysis, synthetic ATCP and ANFT eluted at 4.4 and 6.0 min, respectively (Chart 9A). The product obtained from in vitro incubation of ANFT with mouse liver cytosol preparations, following extraction with diethyl ether and isolation on silica gel column chromatography, showed a component eluting at 4.4 min, identical to that of ATCP (Chart 9B). Furthermore, the mass spectrum (Chart 8B) of the product showed that it was identical to that of chemically synthesized ATCP, both exhibiting the same molecular ion at m/e 181, with similar fragmentation pattern. Since the enzymatically obtained metabolite had chromatographic properties and mass spectra identical to those of the chemically obtained product, they must be identical and thus correspond to ATCP.

**Characterization of Urinary ATCP.** Chart 8C shows the mass spectrum of the metabolite isolated from urine from FANFT-fed mice. The spectrum is similar to that of synthetic ATCP (Chart 8A), exhibiting the molecular ion at m/e 181, along with similar fragmentation patterns. On HPLC, the urinary metabolite showed a peak at a retention time of 4 min (Chart 9C), the same as that observed with synthetic ATCP. TLC of the urinary metabolite demonstrated a spot with a Rf identical to that of ATCP when developed on silica gel ethyl acetate:n-hexane (Rf 0.2) or with benzene:chloroform:methanol (Rf 0.5), and also on cellulose
plates developed with 2% methanol (R, 0.6) or 2% formic acid (R, 0.8). Furthermore, the UV spectrum of the urinary metabolite showed absorption maxima at 222 and 305 nm, the same as that observed with synthetic ATCP (Chart 10). The urinary metabolite and synthetic ATCP exhibited identical chromatographic and spectral characteristics when analyzed under the same conditions. Thus, the metabolite has been identified as ATCP.

Diethyl ether extraction of urine samples collected during 36 hr following i.g. administration of [2-14C]FANFT demonstrated about 8% of the urinary radioactivity in the organic phase. Estimates of radioactivity from TLC scans (Chart 5) revealed that ATCP constituted about 60% of the ether extract. Based on these data, ATCP accounts for about 5% of the urinary metabolites derived from FANFT. The presence of ATCP in urine of animals given FANFT suggests its formation in vivo.

Mutagenicity Tests of ATCP, ANFT, and FANFT. Mutagenic activities of ATCP, ANFT, and FANFT for Salmonella typhimurium TA 100 are presented in Table 2. ANFT and FANFT were mutagenic even at pmol concentrations. ATCP was less mutagenic compared to ANFT and FANFT, requiring about 6.4 x 10^4 or 8.8 x 10^4 greater concentrations, respectively, to provide the same number of his+ revertant colonies.

DISCUSSION

The present studies showed that the majority of radioactivity from [2-14C]FANFT and [formyl-14C]FANFT was excreted in the urine, feces, or CO2 36 hr after i.g. administration to mice. Similar rapid absorption, degradation, and excretion of radioactivity were reported with other 5-nitrofurylthiazole derivatives such as NFT (19), formic acid 2-[4-(5-nitro-2-furyl)-2-thiazolyl]hydrazide (2), and N-[4-(5-nitro-2-furyl)-2-thiazolyl]acetamide (26). The highest concentration of radioactivity after i.g. administration of [2-14C]-FANFT was in the liver and stomach and not in the major target organs (Table 1). Earlier reports (12, 20) on the excretion studies with rats revealed that, after 24 hr, about 78 and 6% of [2-14C]-FANFT were excreted in urine and feces, respectively. In the mouse after 36 hr, approximately 42 and 39% are excreted in urine and feces, respectively (Table 1). The reason for this difference and its significance, if any, to the tissue specificity remain unclear.

The data in Chart 2 show that the deformylation of FANFT in vivo was very rapid, and substantial amounts of radioactivity could be detected even 4 hr after administration of [formyl-14C]-FANFT. The occurrence of the deformylation reaction was established by studies in vitro (Chart 3), and as shown, the mouse liver 10,000 x g supernatant fraction had an enzyme system capable of converting FANFT to ANFT. Prevalence of the deformylation reaction in vivo was confirmed by chromatographic
analysis of urine, revealing the presence of the deformylated product ANFT (Chart 4). While ANFT was present relatively in greater amounts in the urine collected during the 12-hr period following i.p. administration, very little of it could be detected in 36-hr urine samples following i.g. administration. Formation of ANFT from FANFT in vitro and in vivo and the rapid bioconversion of the former suggest that ANFT is a transient metabolite of FANFT. Whether ANFT is a proximate carcinogenic form of FANFT in the genesis of urinary bladder cancer is not clearly understood. When mice were fed diets containing ANFT, at doses stoichiometrically equivalent to doses of FANFT demonstrated previously to cause a nearly 100% incidence of bladder carcinoma, though it was found to induce a high incidence of tumors, they were not of the bladder but of the forestomach (3). This could be due to various physiological or biochemical factors including the distribution of nitroreductase enzymes, such as xanthine oxidase, NADPH-cytochrome c reductase, or aldehyde oxidase (reviewed in Ref. 20), or cooxidative enzyme prostaglandin endoperoxide synthetase (13) between rats and mice. Indeed, in vitro studies showed that ANFT was degraded at a rate of about 29 µmol/g/hr (6.12 mg/g/hr) by mouse liver homogenate under the conditions conducive for activity by nitroreductase enzyme systems (20). This high rate of degradation may also account for the lack of accumulation of large quantities of ANFT in the urine.

The results of the spectroscopic and chromatographic analysis of chemically and enzymatically reduced ANFT clearly established the structural identity of the reduction product to be ATCP. One of the metabolites obtained from urine after administration of FANFT exhibited chromatographic characteristics identical with synthetic ATCP. However, ATCP appears to be a relatively minor metabolic product, since analyses of radioactivity associated with ATCP in the urine extract amounted to about 5% of the administered activity. Furthermore, ATCP might be an innocuous metabolite generated from FANFT, since it is relatively weakly mutagenic (Table 2). Earlier in vitro metabolic studies of FANFT (12) and ANFT (20, 22) demonstrated that these compounds were easily reduced by rat and mouse liver tissues and rabbit kidney microsomes (29). The product of reductive metabolism of FANFT by kidney microsomal nitroreductase has been tentatively identified as an unsaturated ketonitrile (29). Recently, ATCP has been reported to be formed from ANFT with rabbit liver and kidney microsomal preparations (14). However, the carcinogenicity of 5-nitrofurthiazoles in rabbits has yet to be demonstrated. Our observations on the identification of ATCP demonstrate for the first time its formation in vitro and in vivo from FANFT. The formation of ATCP from FANFT involves deamidation and nitroreduction reactions. The evidences for the occurrence of deamidation reaction in vivo and in vitro were discussed earlier. Furthermore, we demonstrated that the deamidated product ANFT undergoes nitroreduction to generate ATCP, possibly through the 5-nitroso-, N-hydroxylaminoo-, or amino- furfurylthiazole intermediates. Earlier, we reported (21) that TCP was generated in vitro on incubation of NFT with rat liver subcellular fractions under anaerobic conditions in the presence of NADPH, and we postulated a pathway for TCP formation. Subsequently, we demonstrated (17) that the amino intermediates are unstable and act as precursors to generate TCP nonenzymatically. In this regard, formation of ATCP from ANFT suggests that a similar pathway involving nitroreduction and furofuran ring cleavage is operative and that it might be a common pathway for other closely related carcinogenic 5-nitrofurthiazole analogues such as N-[4-(5-nitro-2-furyl)-2-thiazolyl]acetamide, 2-methyl-4-(5-nitro-2-furyl)thiazole, etc. Although our results suggest the formation of ATCP from FANFT might involve deamidation first followed by nitroreduction, alternate pathways for the generation of ATCP from FANFT in vivo cannot be excluded at present and need to be further investigated.

The importance of the above nitroreduction pathway in the metabolic activation of and relative tissue susceptibility to carcinogenic 5-nitrofurthiazole remains unclear. We showed earlier, utilizing [2-14C]FANFT and [2-14C]ANFT, that the intermediate(s) produced during the reduction, such as the radicals, 5-nitroso-, N-hydroxylaminoo-, and amino- furans, bind covalently to exogenously added proteins and nucleic acids (22). FANFT, ANFT, NFT, or TCP per se did not show any significant binding, suggesting that the intermediate(s) of reduction are more reactive than the starting materials or terminal biotransformation products. Furthermore, structure-activity relationships for a number of 5-nitrofurans have established that the nitro group is required for the mutagenicity and carcinogenicity of these compounds (7, 9, 11). In addition, bacterial mutation studies on nitrofurazone, nitrofurantoin, furofuramide (AF-2), and metronidazole revealed that the mutants insensitive to these chemicals were also deficient in nitroreductases (10, 15, 16). However, the observed reactivity of the intermediates of reduction, their relationship to the causality of neoplasia, and the metabolic factors involved in the organotropism observed with these 5-nitrofurthiazole analogues remain to be further investigated.

ACKNOWLEDGMENTS

We thank J. Hatcher for mutagenicity assays and K. Bloemstrum and S. Pertzborn for aid with manuscript preparation.

REFERENCES

S. Swaminathan and G. T. Bryan

1397-1401, 1981.


Biotransformation of the Bladder Carcinogen \( N \) \([4-(5-Nitro-2-furyl)-2-thiazolyl]\)formamide in Mice

Santhanam Swaminathan and George T. Bryan

*Cancer Res* 1984;44:2331-2338.

Updated version

Access the most recent version of this article at:

http://cancerres.aacrjournals.org/content/44/6/2331

E-mail alerts

Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.