Metabolism of Nitromiphene (CI 628) in the Immature Female Rat: Role of Gastrointestinal Microflora in the Biotransformation of a Triarylethylene Antiestrogen

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ABSTRACT

Nitromiphene (NIT; CI 628) is a triarylethylene antiestrogen shown to be effective in treatment of experimental breast cancer. We have studied the fate of NIT in the immature female rat, the animal model in which most of the biochemical studies of NIT have been carried out. NIT was eliminated mainly via the feces after i.p. administration, primarily as metabolites. One of these, a diphenylmethane derivative, p-[2-[(N-pyrrolidiny1)ethoxy]-p'-methoxybenzophenone (PMB), was also eliminated in urine as and as its O-demethyl and keto-reduced metabolites. In uterine and liver tissue, unchanged NIT was accompanied by demethyl NIT (CI 628M), PMB, and a diarylacetophenone derivative, p-[2-[(N-pyrrolidinyl)ethoxy]-p'-hydroxybenzhydryl phenyl ketone (demethyl KET). In vitro studies showed that O-demethyl NIT was produced in the presence of liver enzymes and that PMB and demethyl KET were produced in the presence of intestinal bacteria. These results suggested that PMB and demethyl KET accumulate in uterine and liver tissue due to reabsorption from the intestine after having been produced there from NIT and demethyl NIT, respectively. The effects of antiestrogens and their metabolites may be due in part to interaction with antiestrogen binding sites. Both demethyl KET and PMB had good affinity for such sites. Thus, these enteric bacterial metabolites not only have the ability to accumulate in vivo, but could, together with demethyl NIT, contribute to the antiestrogenic effects observed with NIT.

INTRODUCTION

Experimental and clinical applications of triarylethylene antiestrogens are due primarily to the ability of these compounds to antagonize the effects of estrogens (1). One of these, NIT (Fig. 1), has thus been shown to suppress the growth of chemically induced and transplanted mammary tumors in rodents (2, 3). Also, NIT was shown to have potent, prolonged antitumor effects in immature rats (4-6).

There has been considerable interest in determining the molecular mechanism(s) by which NIT and other antiestrogens exert their effects. A complicating factor in mechanistic studies is metabolism. NIT has been shown to undergo conversion to demethyl NIT (CI 628M), a phenolic metabolite which had greater affinity for estrogen receptors and greater biological potency in vitro than did NIT (7, 8). However, the in vivo antiestrogenic effects of NIT and demethyl NIT were similar (8), possibly due to facile O-demethylation of the former compound after administration. Consideration of the structure of NIT with respect not only to oxidative but to reductive routes of metabolism of related compounds led us to consider other possibilities.

In the presence of intestinal bacteria in vitro, NIT underwent facile reduction-hydrolysis, affording KET (9). Also produced, in approximately equal proportion, was a novel "hydrolytic" product, PMB. We thought that NIT might undergo conversion to KET and PMB after parenteral administration, in the event that it underwent extensive clearance into the intestine as does a related antiestrogen, tamoxifen (10). Since the majority of mechanistic studies of NIT have used the immature female rat (5-8), we chose to study its fate in this animal model as well.

During these studies, it became apparent that the postulated metabolites and/or their O-demethylated counterparts were present in significant concentrations in vivo. These compounds each have structural features typical of ligands with high affinity for antiestrogen binding sites. Such sites, which are found in uterus and liver, will bind a rather wide range of nonpolar compounds bearing basic side chains, although maximal affinity appears to be restricted to triarylethenes structurally similar to NIT (11-14). These sites have been suggested to affect the distribution of triarylethylene antiestrogens (11, 15) and could thus affect those of their metabolites as well, providing affinity is retained. Thus, we have evaluated the ability of NIT and its metabolites to interact with hepatic antiestrogen binding sites.

MATERIALS AND METHODS

Chemicals

Demethyl NIT was prepared as described previously (16). PMB, NIT, and NIT N-oxide were available from a previous study (9).

Chemical Methods

Preparation of Demethyl PMB. A solution of 2.0 g (4.3 mmol) of 4-[(2-bromoethoxy)-4'-hydroxybenzophenone (16) and 6 g (84 mmol) of pyridolidine in 25 ml of tetrahydrofuran was stirred and refluxed for 4 h. After concentration in vacuo, the mixture was worked up with ether and 10% aqueous NaOH. The ether layer was concentrated in vacuo. The product was crystallized from benzenevaseline acetate (3:1) and then from methanol to give 0.9 g of white plates, m.p. 159-160°C.

C15H19NO3
Calculated: C 73.37, H 7.70, N 4.28, O 14.66
Found: C 73.25, H 7.71, N 4.23

Preparation of PMB-ol. A solution of 162 mg (0.5 mmol) of PMB in 3 ml of ethanol was stirred with 36 mg (1 mmol) of NaBH4. After 5 h, the mixture was concentrated and the residue was partitioned between ether and saturated aqueous NaCl. The ether phase was concentrated in vacuo to give 0.12 g of a white solid which crystallized from ether-petroleum ether as white clusters, m.p. 83-84°C.

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METABOLISM OF NITROMIPHENE

Fig. 1. Structures of NIT and its metabolites. For convenience, only the "trans" isomer of the triarylethenes is shown, although each of these is composed of both geometric isomers in about equal proportions. In solution, KET and demethyl KET are present as the respective keto tautomers.

Radiochemicals

3,5-[3H]-4-Hydroxytamoxifen (41.3 Ci/mmol) was purchased from Amersham Corp., Arlington Heights, IL. Pyrrolidine-2-[3H]NIT (19.7 mCi/mmol) and [14C]NIT (0.23 mCi/mmol, labeled at the carbon bearing the nitro group) were prepared as described previously (17, 18).

Biochemicals

All biochemicals used in this study were obtained from Sigma Chemical Co., St. Louis, MO. The buffer used in binding site assays was composed of Tris base (10 mM), disodium EDTA (1.5 mM), and sodium azide (3 mM), adjusted to pH 7.4 by addition of 10% HCl. Dextran-coated charcoal suspension was prepared by stirring a suspension of 100 mg of Dextran C and 1 g of Norit A in 100 ml of deionized water for 12 h at 8°C. The mixture was centrifuged and the precipitate was resuspended in 100 ml of cold Tris:EDTA:sodium azide buffer. The mixture was stored at 8°C.

Chromatography

TLC was carried out using 20- x 20-cm plastic backed sheets coated with Silica Gel 60 F254 (EM Reagents Catalog 5775). Plates were developed using solvents of the following compositions, by volume: Solvent 1, chloroform:methanol:28% aqueous ammonia, 90:10:0.5; Solvent 2, benzene:triethylamine, 85:15; Solvent 3, chloroform: methanol:28% aqueous ammonia, 95:5:0.5. Radiochemical purities were determined by TLC using Solvent 1.

Table 1 TLC Rf values of NIT and its metabolites

Conditions for chromatography are described in "Materials and Methods." Chromatograms were inspected under light of 254 nm wavelength. Dual entries indicate partial separation of geometric isomers.

<table>
<thead>
<tr>
<th>Compound</th>
<th>TLC solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>NIT</td>
<td>0.59, 0.65</td>
</tr>
<tr>
<td>Demethyl NIT</td>
<td>0.34</td>
</tr>
<tr>
<td>KET</td>
<td>0.55</td>
</tr>
<tr>
<td>Demethyl KET</td>
<td>0.28</td>
</tr>
<tr>
<td>PMB</td>
<td>0.52</td>
</tr>
<tr>
<td>Demethyl PMB</td>
<td>0.25</td>
</tr>
<tr>
<td>PMB-ol</td>
<td>0.38</td>
</tr>
<tr>
<td>NIT N-oxide</td>
<td>0.13, 0.16</td>
</tr>
</tbody>
</table>

Isotope Dilution Procedure for Metabolite Identification

An aliquot, typically 25–100 μl of the reconstituted tissue, urine, or fecal extract from animals which received [3H]NIT was applied to a TLC plate. To this zone was applied 8–16 μl of a solution of authentic standard compounds in concentrations such that each compound, when eluted from the plate in 1.0 ml of ethanol, would have absorbance of approximately 1.0 at its λmax. The plate was developed with Solvent 1. Authentic standards were located under UV of 254 nm wavelength, cut out, and eluted in separate 1.0-ml aliquots of ethanol for at least 12 h. Total radioactivity (nCi) in each eluate was determined by counting a 0.1-ml aliquot and dividing dpm found by 220. Specific activity was calculated by dividing this value by nmol of standard compound found by UV to be present in solution. The remaining 0.9 ml of each eluate was concentrated and the residue was dissolved in 50 μl of ethyl acetate. Chromatography, elution, and quantitation procedures were carried out as described above. Solvent 2 was used for chromatography; standard compounds were eluted in 0.9-ml aliquots of ethanol. The procedure was repeated a third time using Solvent 3 for chromatography; standards were eluted in 0.8-ml aliquots of ethanol.

Competitive Binding Studies

Incubations were carried out in triplicate at 4°C in 12.5- x 75-mm polypropylene centrifuge tubes, as described (14).

RESULTS

Levels of uterine [3H] were maximal 4 h after administration of [3H]NIT (Table 2). Dilution of [3H] in nuclear pellet extracts with standard compounds, which were then separated by TLC, was used to estimate levels of NIT and its metabolites. NIT was accompanied by PMB and demethyl KET as major metabolites. Demethyl NIT was found in all extracts except the one obtained 24 h after dosing, but at lower levels. As was the case in uterine tissue, levels of [3H] were maximal in the livers of animals 4 h after administration of [3H]NIT.

Experiments with Cecal Contents

Ceca were obtained from animals from which livers had been removed (above). Preparation of cecal content suspensions was carried out as described (9).

In Vivo Experiments

Animals were randomly divided into three groups of four. Each animal received 0.15 mg (approximately 6 μCi) of [3H]NIT, as the citrate salt, i.p. in a volume of 0.25 ml. Each group was housed in a plastic metabolism cage and was allowed unlimited food and water. Two h after dosing, the first group was killed by CO2 asphyxiation. Liver and uteri were removed by dissection and weighed. At 4, 8, 16, and 24 h after dosing, these tissues were obtained similarly, and weight or volume was recorded. Also, urine and feces from the 24-h group were weighed and frozen prior to processing. Processing of biological samples was carried out exactly as described (20), using [14C]NIT citrate as an internal standard to assess efficiency of recovery of total [3H]. The urine extract was concentrated in vacuo and the residue was dissolved in 3 ml of 0.05 M phosphate buffer, pH 7.4. The solution was divided into three equal portions. To the first and second portions were added, in turn, 100 units of sulfatase in 1.0 ml of buffer and 1000 units of β-glucuronidase in 1.0 ml of buffer. All three portions were allowed to stand at room temperature for 24 h. Each was shaken with 2.5 ml of ether for 0.5 h. Aliquots of each of the respective organic and aqueous phases were counted. The organic phases were concentrated and subjected to TLC.
The biotransformation of NIT, demethyl NIT, and PMB was studied under selected in vitro conditions in order to determine the sequence and degree of involvement of liver and intestinal bacterial enzymes. Aerobic incubation of [3H]NIT with liver 9000 x g supernatant fortified with NADPH resulted in 25% consumption of the substrate with respect to control (NADPH-absent) incubations (Fig. 3). This was primarily due to O-demethylation of NIT. Reduction to KET and hydrolysis to PMB did not occur under these conditions or in the absence of oxygen. Exclusion of oxygen resulted only in reduction of substrate consumption and demethyl NIT formation. Chromatograms of extracts from aerobic incubations of PMB with liver 9000 x g supernatant exhibited a single product which had an Rf value identical to that of demethyl PMB, using each of the three TLC solvent systems.

Although NIT was not converted to KET or PMB via liver enzymes, facile enzymatic conversion to these metabolites took place in the presence of intestinal bacteria, as shown in Fig. 3. Similarly, demethyl NIT underwent conversion to a product shown spectrally to be demethyl KET (Table 4); no material with chromatographic Rf equal to that of demethyl PMB was detected.

Like demethyl NIT, PMB and demethyl KET had good affinity for rat liver antiestrogen binding sites, although none of these metabolites had affinity for these sites equal to that of unchanged NIT (Table 5). Relative affinities were determined in competitive binding assays with [3H]-4-hydroxytamoxifen, a high affinity ligand for antiestrogen binding sites (11).

**DISCUSSION**

Two aspects of these studies indicate that intestinal bacteria affect the fate of NIT in the immature rat. First, not only was NIT eliminated mainly as bacterial metabolites in feces, but urine contained PMB and related diphenylmethane metabolites. And PMB was accompanied by demethyl KET in uterine and liver tissue (Tables 2 and 3). Repetitive TLC of hepatic and urinary PMB showed it to be homogeneous, but hepatic demethyl KET contained one or more other components which haven't been structurally characterized (Fig. 2). Secondly, in vitro metabolism experiments showed that bacterial but not hepatic enzymes catalyzed the formation of KET and PMB (Fig. 3). Different bacterial enzymes are evidently involved in the biotransformation of NIT, demethyl NIT, and PMB.

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METABOLISM OF NITROMIPHENE

Table 4 Comparative spectral properties of metabolic demethyl KET and synthetic KET

<table>
<thead>
<tr>
<th>Compound</th>
<th>Demethyl KET</th>
<th>KET</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV (\lambda_{max})</td>
<td>240, 282 nm</td>
<td>232, 273 nm</td>
</tr>
<tr>
<td>Absorbance ratio, (\lambda_{282}/\lambda_{240})</td>
<td>0.24</td>
<td>0.62</td>
</tr>
<tr>
<td>IR (C=O)</td>
<td>1681 cm(^{-1})</td>
<td>1690 cm(^{-1})</td>
</tr>
<tr>
<td>Fast-atom bombardment mass spectral (MH^+ m/e)</td>
<td>402.2071*</td>
<td>416</td>
</tr>
<tr>
<td>Calculated for (C_{20}H_{28}NO_3)</td>
<td>402.2069</td>
<td></td>
</tr>
</tbody>
</table>

* The spectrum was recorded using previously reported conditions and procedures (29).

Table 5 Affinity of NIT and its metabolites for rat liver microsomal antiestrogen binding sites

<table>
<thead>
<tr>
<th>Compound</th>
<th>(IC_{50}) (M)</th>
<th>RBA</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIT</td>
<td>8.2 ± 0.6 x 10(^{-4})</td>
<td>122</td>
</tr>
<tr>
<td>Demethyl NIT</td>
<td>2.6 ± 0.5 x 10(^{-4})</td>
<td>38</td>
</tr>
<tr>
<td>PMB</td>
<td>7.4 ± 1.2 x 10(^{-4})</td>
<td>13.5</td>
</tr>
<tr>
<td>Demethyl PMB</td>
<td>8.6 ± 1.2 x 10(^{-4})</td>
<td>1.2</td>
</tr>
<tr>
<td>PMB-ol</td>
<td>5.5 ± 0.9 x 10(^{-4})</td>
<td>0.2</td>
</tr>
<tr>
<td>KET</td>
<td>1.1 ± 0.4 x 10(^{-4})</td>
<td>90</td>
</tr>
<tr>
<td>Demethyl KET</td>
<td>4.5 ± 0.5 x 10(^{-4})</td>
<td>23</td>
</tr>
</tbody>
</table>

\(\text{RBA} = \frac{\text{IC}_{50} \text{of 4-hydroxy-TAM}}{\text{IC}_{50} \text{of test compound}} \times 100.\)

The \(IC_{50}\) of 4-hydroxy-TAM was 1.0 ± 0.1 x 10\(^{-4}\) M.

formation of these two products since demethyl NIT underwent reduction to demethyl KET, but ethylenic bond cleavage to demethyl PMB was not observed. This finding also suggests that the demethyl KET localized in liver and uterine tissue arises mainly from reduction of demethyl NIT, inferred to be present in the gut (Table 3), rather than via hepatic O-demethylation of KET since KET was detected only at low levels in liver tissue. In summary, the in vitro experiments suggest that NIT biotransformation proceeds as summarized in Fig. 4, and imply that PMB and demethyl KET are “recycled” after clearance of NIT and demethyl NIT into the intestine.

Administration of NIT at a dose of approximately 15% of that used in this study resulted in uterine accumulation of demethyl NIT 1 h after administration (19). Although the current results showed that NIT underwent facile hepatic O-demethylation in vitro (Fig. 3) and in vivo (Table 3), low levels of demethyl NIT were found in uterine nuclear fractions in relation to those of PMB and demethyl KET (Table 2). The “high” dose of NIT used in our studies may have interfered in some way with the accumulation of demethyl NIT. On the other hand, 4-hydroxytamoxifen, a metabolite structurally analogous to demethyl NIT, accumulated in uterine nuclei at prolonged intervals after dosing regardless of whether doses of 150 or 5–15 \(\mu\)g/animal of the parent antiestrogen, tamoxifen, were administered (20–22).

The antiestrogenic effects of NIT and other triarylethenes are thought to result mainly from their interaction with estrogen receptors, in part due to biotransformation to metabolites whose estrogen receptor affinities equal or exceed those of the antiestrogens from which they are derived (23). We showed that KET was not a ligand for estrogen receptors and had no antiuterotropic activity (24). Demethyl KET and PMB also had no estrogen receptor affinity under the same conditions (data not shown). On this basis it would thus appear unlikely that the

Fig. 2. Isotope-dilution TLC analysis of demethyl KET (■); demethyl PMB (○); PMB (■); and PMB-ol (△) in extracts from liver (---) 8 h after dosing and urine (-----) 0–24 h after dosing. The specific activities of PMB-ol at each TLC step were one-tenth of those shown.

Fig. 3. In vitro metabolism of NIT. Recoveries of NIT (■); KET (□); PMB (○), and demethyl NIT (△) were determined after incubation of \([3H]\)NIT with liver 9S fraction, with or without 0.4 mM NADPH under air (NADPH) or nitrogen (NADPH*), or cecal content suspension preincubated for 20 min (25°C) in the absence or presence of 1 mM concentrations of phenylmercuric chloride (PhHgCl), N-ethylmaleimide (NEM) or N-ethylmaleimide plus glutathione (NEM, GSH*).

Calculated for \(C_{20}H_{28}NO_3\)
anticounterodontic effects of NIT are due to its conversion to these metabolites.

However, the affinity of these metabolites for antiestrogen binding sites suggests a possible influence of such interactions not only on pharmacokinetic properties (15), but also on the antiestrogenic effects of NIT. In addition to their presence in uterine, liver, and other normal cells, antiestrogen binding sites are present in human breast cancer cells, such as the MCF-7 cell line (25). The growth-inhibitory effect of tamoxifen on such cells was increased in the presence of equimolar concentrations of NIT in vivo may be due not just to unchanged drug and metabolites. Its effect on MCF-7 cell growth was suggested to be due to factors other than simply increasing the availability of tamoxifen in the immature rat (27). It had affinity for rat liver microsomal antiestrogen binding sites similar to that of PMB (26). This derivative also antagonized the uterotrophic effect of estradiol in the immature rat (27). For factors other than simply increasing the availability of tamoxifen for binding to estrogen receptors by displacement from antiestrogen binding sites. On this basis, the antiestrogen effects of NIT in vivo may be due not just to unchanged drug and demethyl NIT, but to PMB and demethyl KET as well.

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