Pharmacology, Pharmacokinetics, and Metabolism of Acetothiolutamide, a Novel Nonsteroidal Agonist for the Androgen Receptor

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ABSTRACT
The present study characterized the in vitro androgen receptor (AR) binding affinity, in vitro and in vivo pharmacological activity, and in vivo pharmacokinetics and metabolism of acetothiolutamide, a nonsteroidal AR ligand. AR binding was determined by a competitive binding assay. In vitro AR agonist activity was examined by a cotransfection assay. Acetothiolutamide displayed high AR binding affinity ($K_i = 4.9 \pm 0.2 \text{ nM}$) and full agonist activity in the in vitro studies. Next, the androgenic, anabolic, and antiandrogenic activity of acetothiolutamide was evaluated in a castrated immature rat model. In this animal model, acetothiolutamide exhibited an overall negligible androgenic effect, but a statistically significant anabolic effect at high subcutaneous doses. Also, acetothiolutamide demonstrated a noticeable antiandrogenic effect in castrated rats supplemented with testosterone propionate. To understand the causes for the observed disparity between in vitro and in vivo activities, pharmacokinetics and metabolism of acetothiolutamide were studied in male Sprague-Dawley rats. Acetothiolutamide was rapidly cleared from rat plasma (clearance of about 45 ml/min/kg) in a concentration-independent manner after i.v. dosing. Acetothiolutamide was completely absorbed after subcutaneous administration, but not bioavailable after oral dose. In the metabolism study, the unchanged molecule and its metabolites in urine and fecal samples were detected by high-performance liquid chromatography-mass spectrometry. The structures of major metabolites were elucidated with liquid chromatography-tandem mass spectrometry. After i.v. administration, acetothiolutamide was excreted in urine and feces as unchanged drug and a variety of metabolites. Oxidation, deamination, hydrolysis, and sulfate conjugation of phase I metabolites were the major metabolic pathways of acetothiolutamide in rats. Overall, the high plasma clearance of acetothiolutamide, due to its extensive hepatic metabolism, likely contributed to its lack of androgenic activity in vivo.

For decades, steroidal androgens have been used clinically in the treatment of diseases resulting from androgen deficiency (Conway et al., 1988; Wu, 1992; Morley et al., 1993; Nieschlag, 1996). Recently, they have gained attention for their use as hormone replacement therapy of aging men and regulation of male fertility (Conway et al., 1988; Wu, 1992; Morley et al., 1993; Tenover, 1997). Unfortunately, current steroidal androgen preparations, mainly synthesized testosterone and its 17α- and 17β-hydroxyl-modified analogs, have severe limitations, which have compromised their therapeutic success (Wu, 1992). Unmodified testosterone is rapidly degraded by the liver and thus has a low systemic bioavailability after oral administration and a short duration of action after parenteral doses (Handelsman et al., 1990). Esterification at the 17β-hydroxy group of testosterone prolongs its duration of action and enables its practical use by intramuscular injection (Snyder and Lawrence, 1980). However, the resulting large variations and fluctuations in plasma testosterone levels are troublesome. On the other hand, 17α-alkylated testosterones undergo reduced hepatic inactivation and can be given orally. However, they often cause unacceptable hepatotoxicity and are less efficacious (Heywood et al., 1977; Ishaak and Zimmerman, 1987). In addition, steroidal androgens and some of their in vivo metabolites may cross-react with steroid receptors other than the androgen receptor (AR), and accordingly, produce undesirable effects. Great efforts have been made attempting to overcome the drawbacks of androgen preparations. Most studies were focused

**ABBREVIATIONS:** AR, androgen receptor; TP, testosterone propionate; HPLC, high-performance liquid chromatography; LOQ, limit of quantitation; LC-MS, liquid chromatography-mass spectrometry; LC-MS/MS, tandem mass spectrometry; AUC, area under the concentration-time curve; CL, clearance; PEG 300, polyethylene glycol 300; ANOVA, analysis of variance.
on new methods or routes of delivery and chemical modification of available steroidal androgens (Wu, 1992). Nevertheless, success has been somewhat limited.

In recent years, there has been growing interest in the development of nonsteroidal modulators for steroid receptors as therapeutic agents. It has been shown that nonsteroidal ligands can achieve better receptor selectivity than their steroidal counterparts (Jones et al., 1996). Moreover, nonsteroidal ligands allow greater flexibility in structural modifications for optimal physicochemical, pharmacokinetic, and pharmacological properties. For the AR, nonsteroidal antagonists (antiandrogens) are now used clinically (McLeod, 1993); however, nonsteroidal agonists (androgens) have just recently been reported by our laboratories as well as others (Dalton et al., 1998; Edwards et al., 1998; Edwards et al., 1999; Hamann et al., 1999; Higuchi et al., 1999; Zhi et al., 1999) and are still in the early stages of drug discovery. Given the advantages of known nonsteroidal modulators of steroid receptors, the discovery of nonsteroidal androgens provides an opportunity for the development of a new generation of androgens with improved clinical therapeutic index.

Our previous studies identified a group of nonsteroidal androgens derived from two known nonsteroidal antiandrogens, bicalutamide (Fig. 1) and hydroxyflutamide (Dalton et al., 1998). Among that group of compounds, R-1 (Fig. 1), an R-bicalutamide derivative with a thio bridge and a para-chloroacetamido substitution in the aromatic B ring, showed the most potent and most efficacious AR agonist activity. Nevertheless, the electrophilic character of the chloroacetamide group in R-1 raised a concern that it could potentially alkylate many nucleophilic sites in a cellular context. Although Scatchard analysis of AR binding demonstrated that R-1 is a reversible ligand for the AR (Mukherjee et al., 1999), it would be meaningful to further examine the possibility of non-electrophilic analogs of the lead antiandrogen pharmacophores as AR agonists. Therefore, we designed and synthesized acetothiolutamide (Fig. 1), a ligand with an acetamido functional group attached to the aromatic B ring. In this article, we report the results of in vitro characterization, as well as the in vivo pharmacological activity, pharmacokinetics, and metabolism of acetothiolutamide in rats.

### Materials and Methods

**Chemicals.** Acetothiolutamide, R-bicalutamide, and R-iodo-bicalutamide were synthesized in our laboratories as described previously (Mukherjee et al., 1999; Kirkovsky et al., 2000). The purities of synthesized compounds were confirmed by elemental analysis and mass spectrometry. [17α-methyl-[H]Mibolerone ([H]mibolerone, 83.5 Ci/mmol) and unlabeled mibolerone were purchased from PerkinElmer Life Sciences (Boston, MA). Triamcinolone acetonide, phenylmethylsulfonyl fluoride, Tris base, sodium molybdate, dibithiothreitol, dihydrotestosterone, testosterone propionate (TP), and polyethylene glycol 300 (PEG 300, reagent grade) were purchased from Sigma-Aldrich (St. Louis, MO). Hydroxyapatite was purchased from Bio-Rad Laboratories (Hercules, CA). EcoLite (+) scintillation cocktail was purchased from ICN Pharmaceuticals (Costa Mesa, CA). Minimal essential medium, Dulbecco’s modified Eagle’s medium, penicillin-streptomycin, trypsin-EDTA, and LipofectAMINE reagent were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum was purchased from Atlanta Biologicals (Norcross, GA). HPLC grade acetonitrile and ethyl acetate were purchased from Fisher Scientific Co. (Fair Lawn, NJ). Ethyl alcohol USP was purchased from AAPER Alcohol and Chemical Company (Shelbyville, KY).

**Animals.** Male Sprague-Dawley rats were purchased from Harlan (Indianapolis, IN). The animals were maintained on a 12-h light/dark cycle with food and water available ad libitum. All animal studies were reviewed and approved by the Animal Care and Use Committee of the University of Tennessee and conformed to the Principles of Laboratory Animal Care (National Institutes of Health publication 85-23, revised 1985).

**In Vitro AR Binding and Transcriptional Activation.** Cytosolic AR was prepared from ventral prostates of castrated male Sprague-Dawley rats (about 250 g). The binding affinity of acetothiolutamide to the AR preparation was determined and analyzed as described previously (Mukherjee et al., 1996, 1999).

The ability of acetothiolutamide to influence AR-mediated transcriptional activation was examined using a cotransfection system, as described previously (Yin et al., 2003). Briefly, monkey kidney CV-1 cells (American Type Culture Collection, Manassas, VA) were seeded into 12-well tissue culture plates at a density of 2 × 10⁵ cells/well and transiently transfected with 50 ng of a human AR expression construct (pCMVhAR; generously provided by Dr. Donald J. Tindall, Mayo Clinic and Mayo Foundation, Rochester, MN), 1 µg of an androgen-dependent luciferase reporter construct (pMMTV-Luc; generously provided by Dr. Ronald Evans, The Salk Institute, San Diego, CA), and 1 µg of a β-galactosidase expression construct (pSV-β-galactosidase; Promega, Madison, WI) for constitutive expression of β-galactosidase using LipofectAMINE. Cells were treated with increasing concentrations of acetothiolutamide, 1 nM dihydrotestosterone, or vehicle for 48 h. The concentrations of acetothiolutamide tested were 1, 10, 100, and 500 nM. After treatment, the cells were lysed. Cell lysates were then harvested and used for the β-galactosidase assay and luciferase assay. The luciferase activity was normalized according to the measured β-galactosidase activity, as described previously (Dalton et al., 1998). To measure any AR-independent effect, a parallel experiment was included in which cells cotransfected with pMMTV-Luc and pSV-β-β-galactosidase only were treated with 500 nM acetothiolutamide.

**Assay for Pharmacological Activity of Acetothiolutamide in Rats.** Immature male Sprague-Dawley rats weighing 110 to 130 g were randomly distributed into 11 groups of five animals. One day before the treatments, groups 1 through 10 were castrated via an abdominal incision under anesthesia. Group 11 served as an intact (i.e., noncastrated) control. All drugs given to animals were freshly prepared as solutions in PEG 300. For anabolic-androgenic assays, groups 1 to 4 of castrated animals received TP (100 µg/day) or acetothiolutamide (100, 300, or 1000 µg/day), respectively, by subdermal placement of Alzet osmotic pumps (model 2002; Alza, Palo Alto, CA). Group 5 received daily oral doses of acetothiolutamide (1000 µg/day as a suspension in honey) by gavage to assess the feasibility of oral dosing. For antiandrogenic assays, groups 6 through 9 received TP (100 µg/day) by subdermal implantation of Alzet osmotic pumps. Simultaneously, they received 300 µg/day of

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**Fig. 1.** Chemical structures for ligand R-1, R-bicalutamide, and acetothiolutamide. Aromatic rings are designated as A and B as shown.
R-bicalutamide and 100, 300, or 1000 μg/day of acetothiolutamide, respectively, via separate osmotic pumps. Animals in group 10 were implanted with one or two osmotic pumps filled with PEG 300 only. Two weeks after initiation of the treatment, rats were sacrificed. Plasma samples were collected and stored at −20°C. Osmotic pumps were removed, and the residual amount of drug solution in each pump was measured to check for correct pump operation. The ventral prostates, seminal vesicles, and levator ani muscle were removed, cleared of extraneous tissue, and weighed. All organ weights were normalized to body weight. The weights of ventral prostates and seminal vesicles were used as indexes for evaluation of androgenic or antiandrogenic activity, and the levator ani muscle weight was used to evaluate the anabolic activity (Saksena and Chaudhury, 1970; Steinertz et al., 1971).

**Pharmacokinetic Studies.** The pharmacokinetics of acetothiolutamide was determined in male Sprague-Dawley rats weighing approximately 250 g after an intravenous dose at 0.5 or 10 mg/kg body weight, or a subcutaneous or oral dose at 10 mg/kg body weight. Animals for the intravenous study were catheterized in the right external jugular vein and femoral vein and allowed to recover for 24 h. The compound was dissolved in PEG 300 and injected as a bolus dose via the femoral vein cannula. Blood samples (200−300 μl) were collected via the external jugular vein immediately before and at 3, 5, 10, 30, 60, 90, 120, 180, and 240 min after the dose. In the subcutaneous and oral studies, animals were catheterized in the right external jugular and allowed to recover for 24 h. The compound was dissolved in PEG 300 and injected subcutaneously or administered orally by gavage. Blood samples (200−300 μl) were collected via the external jugular vein immediately before and at 10, 20, 30, 60, 90, 120, 180, 240, and 360 min after the dose. In all experiments, blood samples were centrifuged immediately at 2000g, 4°C for 10 min, and plasma fractions were harvested and stored at −20°C until analysis.

**HPLC Analysis.** Aliquots of plasma (100−180 μl) from the pharmacokinetic study were spiked with an internal standard (R-iodobicalutamide) and mixed with 2 volumes of acetone. After centrifugation at 2000g, 4°C for 5 min, the supernatants were adjusted to 1 ml with 0.05 M phosphate buffer 7.0 pH and evaporated to an extent of 6 ml of ethyl acetate. The organic phase was then evaporated, and the residues were reconstituted in 200 μl of mobile phase. An aliquot of each sample was injected onto a Nova-pak C18 column (3.9 × 150 mm, 4-μm particle size; Waters, Milford, MA), and eluted with mobile phase containing acetone/water [40:60 (v/v)] at a flow rate of 1.0 ml/min. The UV absorbance of the elution mixture was monitored at 270 nm. The HPLC system consisted of a model 510 solvent pump (Waters), a model 717 autosampler (Waters), and a SP8480 XR detector (Spectra-Physics, San Jose, CA). Specificity for acetothiolutamide detection in plasma samples was confirmed by the lack of coelution in the compound peak and the identical full-range UV spectrum to that of the pure compound, as determined in separate analyses of several plasma samples from the pharmacokinetic study using a photodiode array detector (model 996; Waters) connected to a model 2690 separations module (Waters). Calibration standards were prepared in drug-free rat plasma with acetothiolutamide concentrations ranging from 0.4 to 100 μg/ml. The recoveries of the compound over the calibration range were from 94.2 to 102.0%. The intra- and inter-day coefficients of variation of the assay were 7.2 and 10.0%, respectively, at 0.4 μg/ml (limit of quantitation, LOQ), and 5.5 and 4.2%, respectively, at 100 μg/ml.

**Rat Plasma Protein Binding.** The binding of acetothiolutamide to rat plasma proteins was determined by an ultrafiltration method using a MPS micropartition device with YMT membrane (Amicon, Beverly, MA). Acetothiolutamide (final concentrations 4 and 20 μg/ml) was added to plasma collected from Sprague-Dawley rats (the final mixture contains 99% plasma) and incubated at 25°C (room temperature) for at least 30 min before centrifugation at 1200g, 25°C for 15 min. R-Lodo-bicalutamide, the internal standard, was added to aliquots of the ultrafiltrate and analyzed for acetothiolutamide concentration using the HPLC method described above. The adsorption of acetothiolutamide to the filtration device and membrane over the concentration range of 0.4 to 40 μg/ml was determined by comparing acetothiolutamide concentrations in deionized water before and after centrifugation in the filtration device at 1200g, 25°C for 3 min.

**Metabolism Studies.** Adult male Sprague-Dawley rats, weighing approximately 250 g, were catheterized in the right external jugular vein and housed in metabolism cages. Before dosing, urine and feces were collected for 24 h. The rats were then given acetothiolutamide via the jugular catheter at a dose of 50 mg/kg. Urine and fecal samples were collected at 24-h intervals for up to 48 h after the dose. The animals were transferred to new cages at the end of each collection interval. All samples were stored frozen at −20°C until analysis.

Before analysis, urine samples were thawed and centrifuged. An aliquot (10 μl) of supernatant from each collection interval was extracted with the same volume of ethyl acetate. The extraction procedure was repeated four times. The combined organic phase and the aqueous phase were evaporated separately to dryness under reduced pressure. The residues were reconstituted in a mobile phase of acetone/H2O (60:40 (v/v)), and the solution was filtered through a membrane filter (0.45 μm; Millipore Corporation). The filtrate was applied to liquid chromatography-mass spectrometry (LC-MS) and tandem mass spectrometry (LC-MS/MS) analyses, as described below.

Fecal samples (6.0−10.5 g) were homogenized with a mechanical homogenizer after adding 10 ml of distilled water. The homogenates were extracted three times with a mixture of methanol/ethyl acetate (2:1 (v/v)). The liquid phase after each extraction was combined and evaporated under reduced pressure. The resulting semisolid residues were then well mixed with methanol, and an aliquot (300 μl) of each methanol extract was filtered through a membrane filter, followed by LC-MS and LC-MS/MS analyses.

**LC-MS and LC-MS/MS Analyses.** LC-MS and LC-MS/MS were performed with a Bruker-Hewlett Packard Esquire-LC system, which consisted of a 1100 HPLC (Hewlett Packard, Palo Alto, CA) coupled to a Bruker mass spectrometer with electrospray interface. Data acquisition was controlled by ChemStation software (Hewlett Packard). An aliquot (20 μl) of each sample was injected onto a Nova-pak C18 column (3.9 mm i.d. × 150 mm length, 4-μm particle size; Waters) and eluted with a mobile phase of acetone/H2O (60:40 (v/v)) at a flow rate of 0.5 ml/min. After detection of the UV absorbance, the filtrate was applied to liquid chromatography-mass spectrometry (LC-MS) and tandem mass spectrometry (LC-MS/MS) analyses, as described below.

**Data Analyses.** The plasma concentration-time data were analyzed by noncompartmental methods using WinNonlin (version 3.1; Pharsight, Mountain View, CA). The area under the plasma concentration-time curve (AUC) was calculated by the trapezoidal rule with extrapolation to time zero. The terminal elimination half-life (t1/2) was calculated from t1/2 = 0.693/λ, where λ was the terminal elimination rate constant. The plasma clearance (CL) was calculated as CL = Dose0−t/AUC 0−t, i.e., where Dose0−t, and AUC0−t, i.e., are the i.v. dose and the corresponding area under the curve from time 0 to infinity, respectively. The apparent volume of distribution at equilibrium (Vss) was calculated using the method of Benet and Galeazzi (1979). Subcutaneous bioavailability (Fa.s.c.) was calculated as follows:

\[ Fa.s.c. = \frac{(AUC_{0-\infty, c}) \cdot (Dose_{iv})}{(AUC_{0-\infty, i.v.}) \cdot (Dose_{iv})} \]
where $D_{\text{sub}}$ and $A_{\text{UC}, \text{sub}}$ were the mean subcutaneous dose and the corresponding mean area under the curve from time 0 to infinity, respectively.

The percentage of acetothiolutamide bound to plasma protein (PB%) was calculated as follows:

$$\text{PB}\% = \left(1 - \frac{A_{\text{free}}}{A_{\text{total}} - A_{\text{adsorption}}}\right) \cdot 100$$

where $A_{\text{total}}$ and $A_{\text{free}}$ were the amount of total acetothiolutamide and free acetothiolutamide at equilibrium, respectively, in the volume of filtered plasma (0.5 ml), and $A_{\text{adsorption}}$ was the amount of acetothiolutamide adsorbed to the filtration device and membrane.

All statistical analyses were carried out by single-factor ANOVA. The $\alpha$ value was set a priori at $p < 0.05$.

Results

In Vitro AR Binding Affinity and Transcriptional Activation

Acetothiolutamide competitively displaced $[3H]$mibolerone from the AR binding site with a mean $K_i$ value of 4.9 ± 0.2 nM. As previously reported, the $K_i$ value of $R$-bicalutamide, a close structural analog and clinically antiandrogen, was 11.0 ± 1.5 nM (Mukherjee et al., 1996). Thus, acetothiolutamide exhibited a higher AR binding affinity than $R$-bicalutamide.

Acetothiolutamide induced AR-mediated transcriptional activation in a concentration-dependent manner. Acetothiolutamide concentrations of 1, 10, 100, and 500 nM induced 4, 50, 73, and 70%, respectively, of the transcriptional activation induced by 1 nM dihydrotestosterone. Control experiments using vehicle only showed no transcriptional activation. The activity of acetothiolutamide was mediated by the AR, because 500 nM acetothiolutamide had no effect on reporter gene expression in the absence of the AR expression plasmid. These results clearly demonstrate that acetothiolutamide functions as an AR agonist in the mammalian cell context. Although less potent than dihydrotestosterone, acetothiolutamide showed an efficacy of transcription activation comparable with dihydrotestosterone.

Evaluation of Pharmacological Activities of Acetothiolutamide in Rats

The identification of acetothiolutamide as an AR agonist by in vitro experiments prompted us to evaluate its pharmacological activity in animals. Consequently, the androgenic, anabolic, and antiandrogenic activity of acetothiolutamide was tested in a castrated immature rat model after 14 days of administration. Preliminary experiments showed that acetothiolutamide was stable in PEG 300 at 37°C for at least 14 days (data not shown). Performance of osmotic pumps was validated from parallel positive androgen and antiandrogen control experiments, as well as volumes of residual drug solution at the end of delivery.

The results of the androgenic and anabolic assay are shown in Fig. 2A. Consistent with previous literature reports (Saksena and Chaudhury, 1970; Teutsch et al., 1994; Battmann et al., 1998), castration resulted in a significant reduction in the weights of ventral prostate, seminal vesicles, and levator ani muscle in rats. A subcutaneous dose of TP at 100 µg/day increased the weights of ventral prostate, seminal vesicles, and levator ani muscle in castrated animals by 15.4-, 9.3-, and 2.5-fold, respectively. Acetothiolutamide caused a small but statistically significant increase in the weight of prostates after the tested subcutaneous and oral doses. However, this statistical significance is not likely to convey any pharmacological meaning based on the magnitude of the effect. The fact that acetothiolutamide had no effect on the weight of...
seminal vesicles further indicated a lack of androgenic activity of acetothiolutamide in this rat model. Acetothiolutamide significantly increased the weight of levator ani muscle in castrated rats at higher subcutaneous dose rates (300 and 1000 μg/day), suggesting possible anabolic activity. Again, this effect was much smaller than that observed with TP treatment. In contrast, no effect on the muscle weight was observed after a high oral dose of acetothiolutamide. In the antiandrogenic assay, R-bicalutamide at 300 μg/day repressed TP-induced increases in prostate, seminal vesicle, and levator ani muscle weights by 78, 79, and 50%, respectively (Fig. 2B). Surprisingly, acetothiolutamide was able to partly offset the TP-induced increase in prostate weight in a dose-dependent manner and noticeably antagonize the effects of TP on weights of seminal vesicles and levator ani muscle.

Pharmacokinetics of Acetothiolutamide in Rats

One of the possible explanations for the observed inconsistency between the in vitro and in vivo functional activity of acetothiolutamide was that the plasma concentrations of acetothiolutamide in animals were insufficient to produce a significant degree of androgenic activity. Low subcutaneous bioavailability and/or a high plasma clearance could result in low plasma drug concentrations. To examine this possibility, the pharmacokinetics of acetothiolutamide was characterized after i.v. and subcutaneous administration to male rats.

After single i.v. bolus doses at 5 and 10 mg/kg, the plasma concentrations of acetothiolutamide declined rapidly in an apparent biexponential manner and were below the LOQ after 60 and 90 min, respectively (Fig. 3). The half-lives were between 5- and 10-mg/kg dose levels, suggesting that acetothiolutamide was quickly eliminated from the body after subcutaneous administration.

After subcutaneous dosing at 10 mg/kg, the plasma concentrations of acetothiolutamide increased gradually with a mean C_{max} (± standard deviation) of 1.58 ± 0.58 μg/ml observed at 105 ± 17 min (T_{max}) and then declined monoxponentially with a t_{1/2} of 86.6 min (Fig. 3; Table 1). This t_{1/2} was significantly longer than those measured after i.v. doses (p < 0.001), indicating a "flip-flop" characteristic of acetothiolutamide absorption after subcutaneous dosing. The AUC of acetothiolutamide after subcutaneous dosing (298 ± 61 μg · min/ml) was not significantly different (p = 0.12) from that observed after intravenous dosing (229 ± 54 μg · min/ml), indicating that subcutaneous absorption of the compound was complete and justifying subcutaneous dosing as a viable route of administration for examination of the pharmacological activity of acetothiolutamide in rats.

The pharmacokinetics of acetothiolutamide after a single oral dose was also examined to determine its oral bioavailability. After a 10-mg/kg oral dose, the plasma concentrations of acetothiolutamide were below the LOQ. Hence, acetothiolutamide was not bioavailable after oral dosing, suggesting rapid hepatic and/or gastrointestinal metabolism and/or poor absorption from the gastrointestinal tract.

Plasma Protein Binding

Acetothiolutamide was highly bound (>90%) to rat plasma proteins. The percentage bound was independent of acetothiolutamide concentration over the range from 4 to 20 μg/ml, with 91.9 ± 0.5 and 91.8 ± 1.2%, respectively, of the drug bound to plasma proteins at these concentrations.

Metabolism of Acetothiolutamide in Rats

Metabolite profiles of acetothiolutamide in the urine and feces of rats after a high i.v. dose of acetothiolutamide were generated by electrospray ionization-mass spectrometry analysis of the samples, after a low-resolution separation by reversed phase HPLC. For urine samples, the organic solvent extract and the aqueous fraction after extraction were both analyzed. For fecal samples, the extracted fecal homogenate was analyzed. The mass spectra of postdose samples were compared with those of blank samples obtained before drug administration to identify drug-related components. Molecular ions of interest were then subjected to LC-MS/MS analysis for structural elucidation.

Profiling and Identification of 0- to 24-h Urinary Metabolites. Fig. 4A shows the negative ion mass spectrum of the organic extract of the 0- to 24-h urine sample. In addition to the molecular ion (i.e., [M – H]⁻ deprotonated molecular anion) corresponding to the parent compound (m/z 436), five metabolite ions were detected at m/z 452, 468, 484,
522, and 548, respectively. The molecular ion at m/z 452 represented the base peak in the spectrum, whereas molecular ions at m/z 484, 522, and 548 had a relative ion abundance of less than 20%. All drug-related molecular ions were subsequently characterized by LC-MS/MS.

Molecular ion at m/z 436 ([M−H]− of the parent compound). The theoretical mass of the parent compound is 437. As shown in Table 2, the corresponding [M−H]− ion yielded prominent fragment ions at m/z 418, 269, 250, 222, 213, 185, and 166. The fragment ion at m/z 418 corresponded to the loss of a H2O molecule from the [M−H]− ion, presumably by eliminating the tertiary hydroxy group of the parent compound and forming a double bond between the chiral carbon and the methylene carbon to increase the resonance stability. The overall fragmentation pattern confirmed that the ion at m/z 436 was the deprotonated molecular anion [M−H]− of the parent compound.

Molecular ion at m/z 452 ([M−H]− of mono-oxidized acetothiolutamides). This [M−H]− ion was 16 Da greater than that of the parent compound and corresponded to the addition of an oxygen atom (Table 2). There are three possible ways to add an oxygen atom to acetothiolutamide: 1)
hydroxylation of the aromatic A-ring, 2) oxidation of the sulfur linkage to form a sulfoxide, or 3) hydroxylation of the aromatic B-ring. The presence of fragment ions at \( m/z \) 269, 255, and 212 and the absence of their corresponding oxidized ions in the product ion mass spectrum demonstrated that oxidation did not occur in the A-ring. However, the fragmentation results provided no further information to definitively assign the oxygen atom to either the sulfur or the B-ring. The observation of two closely eluting peaks with the same ion at \( m/z \) 452 in the LC-MS profile (data not shown) and information from metabolites with multiple oxidizations (e.g., the detection of triple-oxidized acetothiolutamide) suggested that the sulfoxide metabolite and the B-ring-hydroxylated metabolite were present in urine. Nevertheless, for the hydroxylated metabolite, the hydroxylation position in B-ring was uncertain.

Molecular ion at \( m/z \) 468 (\([M-H]^−\) of double-oxidized acetothiolutamides). This \([M-H]^−\) ion corresponded to the addition of two oxygen atoms to the parent compound (Table 2). The fragment ions at \( m/z \) 255 and 212 suggested that no oxidation occurred within the aromatic A-ring. There are at least two structural possibilities to yield this molecule ion: one is the sulfone metabolite (two oxygen atoms are added to the sulfur linkage), and the other is the sulfoxide metabolite with a hydroxylated B-ring (one oxygen atom is added to the sulfur linkage, and the other oxygen atom is added to the

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### TABLE 2
Product ions of acetothiolutamide and its metabolites

<table>
<thead>
<tr>
<th>Molecular Ions [M-H]−</th>
<th>Product Ions Observed (m/z Values)</th>
<th>Assigned Structure and Proposed Fragmentation Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>436 (Acetothiolutamide)</td>
<td>436, 418, 355, 288, 269, 250, 222, 213, 191, 185, 166</td>
<td>![Fragmentation Pattern 1]</td>
</tr>
<tr>
<td>452 (Acetothiolutamide + O)</td>
<td>452, 434, 410, 294, 269, 267, 255, 212</td>
<td>![Fragmentation Pattern 2]</td>
</tr>
<tr>
<td>468 (Acetothiolutamide + 2O)</td>
<td>468, 450, 318, 282, 255, 212</td>
<td>![Fragmentation Pattern 3]</td>
</tr>
<tr>
<td>484 (Acetothiolutamide + 3O)</td>
<td>466, 298, 271, 255, 212, 185, 184</td>
<td>![Fragmentation Pattern 4]</td>
</tr>
<tr>
<td>522</td>
<td>522, 504, 442, 294, 255, 212</td>
<td>![Fragmentation Pattern 5]</td>
</tr>
<tr>
<td>548</td>
<td>548, 468, 450, 318, 287, 277, 255</td>
<td>![Fragmentation Pattern 6]</td>
</tr>
<tr>
<td>426</td>
<td>408, 345, 277, 255, 212, 201, 185</td>
<td>![Fragmentation Pattern 7]</td>
</tr>
<tr>
<td>490</td>
<td>491, 490, 472, 451, 410, 394, 304, 285, 276, 255, 225, 201, 185</td>
<td>![Fragmentation Pattern 8]</td>
</tr>
<tr>
<td>564</td>
<td>564, 546, 519, 504, 484, 452, 429, 271, 255</td>
<td>![Fragmentation Pattern 9]</td>
</tr>
</tbody>
</table>

* Denotes a base peak in the product ion mass spectrum.
B-ring). The fragment ion at \( m/z \) 318 (weak) confirmed the formation of the latter metabolite (the sulfoxide metabolite with a hydroxylated B-ring) in urine. However, the formation of the sulfone metabolite could not be excluded.

**Molecular ion at \( m/z \) 484 ([M – H]⁻ of triple-oxidized acetothiolutamide).** This [M – H]⁻ ion corresponds to the addition of three oxygen atoms to the parent compound (Table 2). No deprotonated molecular ion [M – H]⁻ was observed in the product ion mass spectrum. The fragment ion at \( m/z \) 466 resulted from the loss of a \( \text{H}_2\text{O} \) molecule from the molecular ion. The fragment ions at \( m/z \) 255, 212 (weak), and 185 suggested that there was no oxidation in the aromatic A-ring; those data confirmed the observations with the mono- and double-oxidized metabolites. Thus, this molecular ion was most likely produced by the metabolite with a sulfone linkage and a hydroxylated B-ring.

**Molecular ion at \( m/z \) 522 ([M – H]⁻ of B-ring hydrolyzed triple-oxidized acetothiolutamide sulfate conjugate).** As shown in Table 2, this [M – H]⁻ ion gave product ions at \( m/z \) 504, 442, 255, and 212. The loss of 80 Da (that mass corresponds to \( \text{SO}_3 \)) from the molecular ion at \( m/z \) 522 gave the base fragment ion at \( m/z \) 442 suggested that this metabolite was a sulfate conjugate. The fragment ions at \( m/z \) 255 and 212 were also observed in the product ion mass spectra of previous oxidized metabolites. The metabolite for this molecular ion was likely formed from the triple-oxidized metabolite (\( m/z \) 484) by a hydrolysis of the amide bond in the B-ring para-acetamido group (forming an amine group in the B-ring) and sulfate conjugation of either the hydroxy group in the B-ring or the tertiary hydroxy group linked to the chiral carbon.

**Molecular ion at \( m/z \) 548 ([M – H]⁻ of double-oxidized acetothiolutamide sulfate conjugate).** This [M – H]⁻ ion yielded prominent product ions at \( m/z \) 468, 450, and 255 (Table 2). The loss of a \( \text{SO}_3 \) moiety (80 Da) from the molecular ion afforded the base fragment ion at \( m/z \) 468, indicating that this metabolite was a sulfate conjugate. The subsequent loss of a \( \text{H}_2\text{O} \) molecule (18 Da) from \( m/z \) 468 yielded the fragment ion at \( m/z \) 450. The metabolite for this molecular ion was likely produced from the double-oxidized metabolites (\( m/z \) 468) by sulfate conjugation at either the hydroxy group in the B-ring or the tertiary hydroxy group linked to the chiral carbon.

In the aqueous phase after extraction, no drug-related substance was detected in the 0- to 24-h urine sample.

**Profiling and Identification of 24- to 48-h Urinary Metabolites.** The mass spectrum of the organic extract of the 24- to 48-h urine sample is shown in Fig. 4B. The absolute ion abundance of the base peak at \( m/z \) 468 was only 6% of the base peak in the spectrum of Fig. 4A. The parent compound molecular ion at \( m/z \) 436 was still detectable. Besides the molecular ions at \( m/z \) 436, 452, 468, and 522, which were also observed in the first 24-h urine sample, additional drug-related molecular ions were detected at \( m/z \) 426, 490, and 564. Characterization of molecular ions at \( m/z \) 436, 452, 468, and 522 by LC-MS/MS analysis generated product ion mass spectra that were very similar to those described in the preceding section. This similarity indicated that these molecular ions were structurally the same as those in the first 24-h urine sample. The structural identity of molecular ions with the same \( m/z \) ratio in the two urinary samples was also supported by the matched HPLC retention times (data not shown). The LC-MS/MS characterization of molecular ions at \( m/z \) 426, 490, and 564 is described as follows.

**Molecular ion at \( m/z \) 426 ([M – H]⁻ of B-ring hydrolyzed double-oxidized acetothiolutamide).** The product ions of this [M – H]⁻ ion are shown in Table 2. No deprotonated molecular ion [M – H]⁻ was detected under the applied fragmentation conditions. The loss of a \( \text{H}_2\text{O} \) (18 Da) from the molecular ion produced the fragment ion at \( m/z \) 408. The metabolite that corresponded to the [M – H]⁻ ion was likely formed from the double-oxidized metabolites (\( m/z \) 468) by hydrolysis of the amide bond in the B ring para-acetamido group to form an amino group. The fragment ions at \( m/z \) 255 and 212 were also detected in the product ion mass spectrum of the molecular ion at \( m/z \) 468. Like the double-oxidized metabolites, there were at least two structural possibilities for this molecular ion.

**Molecular ion at \( m/z \) 490 ([M – H]⁻ of B-ring hydrolyzed mono-oxidized acetothiolutamide sulfate conjugate).** As shown in Table 2, this [M – H]⁻ ion gave rise to the base fragment ion at \( m/z \) 472 by the loss of a \( \text{H}_2\text{O} \) molecule. The loss of 80 Da from molecular ion at \( m/z \) 490 to produce the fragment ion at \( m/z \) 410 indicated that this metabolite was a sulfone conjugate. The metabolite for this molecular ion was presumably the hydrolyzed and sulfate-conjugated product of the mono-oxidized metabolite (\( m/z \) 452). The hydrolysis occurred at the amide bond of the para-acetamido group in B-ring. The sulfate conjugation occurred at either the tertiary hydroxy group linked to the chiral carbon, or in the B-ring hydroxylated product, the hydroxy group of the B-ring. The fragment ions at \( m/z \) 185 and 255 confirmed that there was no oxidation in the A-ring. In addition, the fragment ion at \( m/z \) 304 indicated the presence of a metabolite with hydroxylination in B-ring, but no oxidation in other positions. However, other structural possibilities could not be excluded.

**Molecular ion at \( m/z \) 564 ([M – H]⁻ of triple-oxidized acetothiolutamide sulfate conjugate).** The product ions of this [M – H]⁻ ion are shown in Table 2. The fragment ion at \( m/z \) 546 corresponded to the loss of a \( \text{H}_2\text{O} \) molecule. The fragment ion at \( m/z \) 484 represented the loss of a sulfate group (80 Da) from the molecular ion at \( m/z \) 564. This fragmentation suggested that this was a sulfate conjugate. The metabolite for this molecular ion was putatively assigned as the sulfate conjugate of the triple-oxidized metabolite (\( m/z \) 484). The sulfate was added to either the tertiary hydroxy group linked to the chiral carbon, or to the hydroxy group at the B-ring.

Again, LC-MS analysis did not detect any drug-related substance in the aqueous phase after extraction of the 24- to 48-h urine sample.

**Profiling of 0- to 24-h Fecal Metabolites.** The mass spectrum of the organic extract of the 0- to 24-h fecal sample is shown in Fig. 4C. The molecular ions that corresponded to the parent compound (\( m/z \) 436), the putative mono-oxidized metabolites (\( m/z \) 452), and the putative double-oxidized metabolites (\( m/z \) 468) were the three major peaks. The molecular ions at \( m/z \) 484 (corresponding to the putative triple-oxidized acetothiolutamide) and 522 (corresponding to the putative B-ring hydrolyzed triple-oxidized acetothiolutamide sulfate conjugate) were also observed with very low ion abundance (less than 5% of the base peak). These molecular ions displayed similar HPLC retention times as corresponding molecular ions obtained in the urine samples (data not shown).
Compared with ligand derivative of the known antiandrogen bicalutamide, was agonist activity (Dalton et al., 1998), acetothiolutamide, a modifications of nonsteroidal antiandrogens can result in metabolites in feces indicated that they were excreted in bile. The appearance of the parent molecule and its metabolites in feces indicated that they were excreted in bile. The formation of antagonistic metabolites of acetothiolutamide (McKillop et al., 1995), the plasma clearance of R-bicalutamide should be even lower than 0.8 ml/min/kg. Although a difference in the rat strain investigated complicates a direct comparison, acetothiolutamide apparently has a much greater clearance than the lead compound R-bicalutamide. The high clearance of acetothiolutamide likely led to insufficient plasma concentrations in animals in the pharmacology studies, and consequently, masked the inherent functional activity of the compound. In the pharmacological studies, the plasma concentrations of acetothiolutamide at the end of delivery were not quantitated, because the steady-state plasma concentrations of acetothiolutamide achieved, as predicted from the pharmacokinetic parameters, would be substantially below the LOQ of the HPLC method.

As shown in the pharmacokinetic studies, acetothiolutamide was completely bioavailable after subcutaneous dosing, thereby excluding incomplete absorption as a cause of its lack of androgenic activity in the animal model. Over the dose range examined, acetothiolutamide was rapidly eliminated from the rat plasma after i.v. dosing with a clearance of about 45 ml/min/kg and a terminal half-life of less than 30 min. The plasma clearance and half-life of bicalutamide, a racemic mixture of R- and S-enantiomers, in rats was 0.8 ml/min/kg and about 1 day, respectively, at a dose range from 0.5 to 2 mg/kg (Cockshott et al., 1991). Considering that R-bicalutamide is cleared much more slowly than S-bicalutamide (McKillop et al., 1995), the plasma clearance of R-bicalutamide was extensive in vivo pharmacological studies.

In accordance with our previous finding that structural modifications of nonsteroidal antiandrogens can result in agonist activity (Dalton et al., 1998), acetothiolutamide, a derivative of the known antiandrogen bicalutamide, was identified as an AR agonist by an in vitro functional assay. Compared with ligand R-1, the most potent nonsteroidal AR agonist previously identified in our laboratory (Dalton et al., 1998), acetothiolutamide demonstrated a similar degree of agonist activity, but does not contain the electrophilic chloroacetamido group. Thus, acetothiolutamide was carried forward to in vivo pharmacological studies.

Inconsistent with the in vitro results, acetothiolutamide showed an overall negligible androgenic effect in a castrated immature rat model with dose rates up to 1000 μg/day. Theoretically, the lack of androgenic activity in vivo could be caused by one or a combination of the following reasons: 1) an insufficient drug concentration at the sites of action in animals due to undesirable pharmacokinetic properties of the compound, for example, a low systemic bioavailability, a high clearance, and/or a low tissue permeability; 2) interference from metabolites formed in animals that may function as AR antagonists; and/or 3) an attenuated inherent AR agonist activity of the compound in rat cells, where the cellular environment could differ from in vitro cultured CV-1 cells, leading to cell-type specific androgenic actions. It is noteworthy that acetothiolutamide did cause a slight increase in the prostate weight, although it had no effect on the seminal vesicle weight. This observation suggests that the drug concentration available at the effector sites in animals might have been in the critical range that can trigger effects on prostate but was below the threshold for effects on seminal vesicles, knowing that ventral prostates are more sensitive to androgens (Hershberger et al., 1953).

Although acetothiolutamide failed to produce androgenic activity in castrated rats, it significantly increased the levator ani muscle weight at high subcutaneous dose rates. This could indicate a preferential anabolic activity of acetothiolutamide per se, an effect mediated through the AR in the muscle. However, it cannot be excluded that acetothiolutamide increased the muscle weight through inhibition of the catabolic effects of glucocorticoids, a mechanism previously postulated for anabolic steroids (Mayer and Rosen, 1975). Furthermore, acetothiolutamide exhibited a small degree of antiandrogenic activity in rats. As detailed in the following discussion, this confounding observation could be caused by the formation of antagonistic metabolites of acetothiolutamide.

Discussion

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The high clearance of acetothiolutamide, as well as its undetectable oral bioavailability, suggested its intensive hepatic metabolism. Thus, the metabolism of acetothiolutamide in male rats was investigated through mass spectrometric characterization of urinary and fecal metabolites. A variety of phase I and phase II metabolites of acetothiolutamide were detected in the biological excreta. These metabolites indicated that acetothiolutamide was extensively metabolized in rats through three major metabolism pathways: oxidation, hydrolysis of the amide bond linked to the aromatic B-ring, and sulfate conjugation. Combining the results from structural characterization, a possible metabolic scheme for the metabolism of acetothiolutamide in rats is proposed and illustrated in Fig. 5. At the early stage of phase I metabolism, acetothiolutamide was oxidized either at the bridge sulfur atom to form sulfoxide, or at the aromatic B-ring to form hydroxylated metabolites. Additional oxidation led to the formation of a sulfone metabolite or a sulfoxide metabolite with B-ring hydroxylation. Eventually, triple-oxidized metabolites were produced. During the oxidation process, the amide bond of the para-acetamido group in the B-ring also underwent hydrolysis, resulting in metabolites with a para-amine group linked to the B-ring. Metabolites formed at various stages were then subjected to sulfate conjugation. It is noteworthy to point out that, although one isomer was indicated for each metabolite in the metabolic scheme, the possibility for the formation of diastereomeric metabolites could not be ruled out.

Previous metabolism studies with bicalutamide showed that cleavage of its amide bond, which corresponds to the A-ring-linked amide bond in acetothiolutamide, was a major metabolism pathway in rats (Boyle et al., 1993), whereas for acetothiolutamide, hydrolysis was only observed at the amide bond of the para-acetamido group in the B-ring, and the A ring-linked amide bond seemed to be stable. Furthermore, it was observed that many oxidized metabolites of
Acetothiolutamide were not hydrolyzed at the B ring-linked amide bond, and almost all detected hydrolyzed metabolites had been oxidized at another site in the molecules. These observations suggested that oxidation was a faster process than hydrolysis.

Sulfate conjugation was the only detected phase II metabolism pathway for acetothiolutamide. Glucuronidation, a major conjugation pathway for bicalutamide in rats (Boyle et al., 1993), was not seen with acetothiolutamide. Sulfate conjugation of acetothiolutamide metabolites could possibly occur at either the hydroxy group linked to the chiral carbon or the hydroxy group introduced into the B-ring during oxidation. Sulfate conjugates were only observed for those oxidized metabolites. No sulfate conjugate of the parent molecule was detected, even in the presence of a high concentration of parent compound. Thus, it is likely that sulfate conjugation occurred at the hydroxy group of the B-ring.

Extensive hepatic metabolism apparently contributed to the high clearance and the lack of androgenic activity of acetothiolutamide in vivo. Previous in vitro structure-activity relationship studies in our laboratories showed that either oxidation at the sulfur linkage or conversion of the acetamido group in the para-position of B-ring to an amine group led to decreased AR binding and agonist activity and enhanced antagonist activity (Yin et al., 2003). Thus, many of the metabolites of acetothiolutamide could be potentially inactive as androgens or active as antiandrogens. The observation of antiandrogenic activity of acetothiolutamide in the pharmacology study was thus not surprising. It is obvious from the proposed metabolic pathways of acetothiolutamide that oxidation was the main theme of metabolism. Therefore, future redesign of structures would focus on circumventing enzymatic oxidation, and in particular, oxidation at the sulfur linkage. Recently completed studies in our laboratories support this hypothesis and are the subject of a forthcoming manuscript.

In conclusion, acetothiolutamide demonstrated potent in vitro androgenic activity, identifying it as a member of a promising new class of nonsteroidal androgens. The lack of in vivo pharmacologic activity of acetothiolutamide was due to its extensive hepatic metabolism, which led to the rapid clearance of the drug from the body.

References


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