

Enzymatic Conversion of the Antibiotic Metronidazole to an Analog of Thiamine¹

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We propose that adverse effects of the antibiotic metronidazole may be due, wholly or in part, to its conversion to a thiamine analog and consequent vitamin B₁ antagonism. Consistent with this hypothesis, the drug is accepted as a substrate for the thiaminase (EC 2.5.1.2) elaborated as an exoenzyme by the human gut flora constituent *Bacillus thiaminolyticus* and is also a substrate for the intracellular thiaminase of the mollusk *Venus mercenaria*. The product, identified as the 1-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-3-(2-hydroxyethyl)-2-methyl-4-nitroimidazolium cation, is a close structural analog of thiamine and is an effective inhibitor of thiamine pyrophosphokinase *in vitro*. Due to its susceptibility to nucleophilic attack, the analog is unstable, releasing inorganic nitrite under mild conditions. Enzymatic alkylation reactions such as that effected by thiaminase may have general pharmacological significance as a route of increasing the electrophilicity and/or reduction potential of drugs which are heterocyclic weak bases.

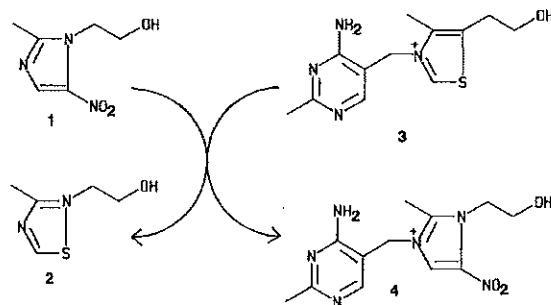
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Metronidazole (1, Scheme I) is valuable in the prevention and treatment of infections by both eukaryotic and prokaryotic anaerobes (1). It has also been advocated for other purposes including chemosensitization and radiosensitization of neoplasms (2, 3) and ethanol deterrence (4). Chronic use of the drug for conditions such as Crohn's disease and acute high-dose use of the drug are limited by peripheral and central adverse neurological effects such as sensory loss, cerebellar symptoms, and convulsions (3, 5-13). The mechanism of neurotoxicity is not established, although histological changes observed in metronidazole-treated rodents have been likened to those seen in states of hypovitaminosis

B₁ (14). Since 1 structurally resembles the thiazole fragment (2) of the thiamine molecule (3), it occurred to us that the drug might form a thiamine analog by replacing the thiazole fragment of the vitamin and that the analog could act as a B₁ antagonist. Formation of the thiamine analog could be catalyzed by a thiaminase (EC 2.5.1.2) as shown in Scheme I. Thiaminases catalyzing transfer of the (4-amino-2-methyl-5-pyrimidinyl)methyl group of thiamine to co-substrates are widely distributed in nature (15, 16) and, although not conclusively demonstrated in mammalian tissues (16-18), are elaborated as exoenzymes by bacteria found among the human intestinal flora. We tested the ability of the drug to function as a substrate for the enzyme reaction shown in Scheme I. Although *N*-methylimidazole (p*K* 7.3) is known to be accepted as a substrate by thiaminase (19), it was not clear a priori that the strongly electron-withdrawing nitro substituent would permit the weakly basic 1 (p*K* 2.7) to participate in the thiaminase reaction.

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SCHEME I. Thiaminase action on metronidazole.

EXPERIMENTAL PROCEDURES

Metronidazole was obtained from the Aldrich Chemical Company. Its pK value was determined by potentiometric and spectrophotometric titration with HCl in 1.0 M KCl at 25°C. Amersham supplied the [thiazole-2-¹⁴C]thiamine. Optical experiments were performed with a Perkin-Elmer 559 recording spectrophotometer. A Varian XL-300 instrument provided NMR spectra. Radioactivity was measured with a Beckman LS-100C photometer employing Amersham ACS scintillant. High-performance cation-exchange chromatography was performed with a Pharmacia FPLC apparatus equipped with a 0.5 × 5.0-cm Mono S column. High-performance liquid chromatography was performed with a Beckman apparatus equipped with a 0.4 × 30-cm Waters μ Bondapak C₁₈ column.

Enzymes. *Bacillus thiaminolyticus* (ATCC 13023) was cultured in Difco nutrient broth in rotary shaker flasks for 3 days at 37°C, and thiaminase was isolated from the centrifugally cell-free broth (20). Thiaminase from locally obtained quahog clams (*Venus mercenaria*) was purified 20-fold (21). Spectrophotometric assays were performed at 319 nm using thiamine and quinoline as substrates (19). Radiometric assays (22) were performed by adding the enzymes to 0.4 ml of a solution containing 50 μ M [thiazole-2-¹⁴C]thiamine and various concentrations of 1 in 200 mM sodium phosphate buffer at pH 6.0 and 25°C. After 15 min, the liberated [¹⁴C]thiazole was extracted into 1.0 ml of ethyl acetate, and 0.6 ml of the organic phase was counted in 4.0 ml of scintillant.

Thiamine pyrophosphokinase was partially purified from autolyzed Sexton baker's yeast by means of heat treatment, acetone precipitation, and ammonium sulfate fractionation (23). The crude enzyme was then purified by the affinity chromatography procedure described for rat brain (24) except that a higher concentration of thiamine, 1.0 mM, was required to elute the yeast enzyme from the thiamine monophosphate-Sepharose column. Assays (25) were initiated by adding the enzyme to 0.2 ml of a solution containing 80–350 μ M [¹⁴C]thiamine, 2.6 mM MnSO₄, 2.6 mM ATP, and 130 mM sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate at pH 7.1 and 25°C. After 20 min, the

reaction mixtures were filtered through 0.5 × 1.5-cm columns of Amberlite CG-50 (H⁺) resin. The columns were washed twice with 0.5 ml of water and the filtrates were counted in 12 ml of scintillant.

Isolation of the product of thiaminase action on the drug. Reaction mixtures containing 20 mM 1 and 1.0 mM thiamine chloride · HCl at pH 4.5 and 25°C were initiated with enzyme (40 μ g/ml) prepared by dialysis against water. Deproteination was effected by high-pressure ultrafiltration through an Amicon PM-10 membrane. In order to obtain NMR spectra, 10 ml of ultrafiltrate was loaded onto a 0.6 × 7.0-cm column of Amberlite CG-50 (H⁺) resin at 4°C. The column was washed with water until the effluent ceased to absorb at 320 nm, and fractions were collected as the bound material was eluted with aqueous formic acid, pH 2.1. Fractions containing 4 were lyophilized, and spectra were recorded immediately upon dissolving the residue in D₂O. Residual 3 was incompletely resolved from 4 on Amberlite columns so that NMR spectra of a 4:1 mixture of product and starting material were recorded: NMR (D₂O) 9.4 (s, 1, imidazolium), 8.1 (s, 1, pyrimidine), 5.4 (s, 2, methylene bridge), 4.7 (t, 2, -CH₂OD), 4.0 (t, 2, =N-CH₂-), 2.9 (s, 3, CH₃-imidazolium), and 2.5 ppm (s, 3, CH₃-pyrimidine).

For electronic spectra, 4 was completely resolved from 3 on the Pharmacia Mono S column (0.5 × 5.0 cm). When the column was eluted with 30 ml of 20 mM sodium acetate buffer, pH 4.5, containing 0 to 500 mM NaCl in a linear gradient, 3 was found in the 250 mM NaCl fraction while 4 was found in the 350 mM NaCl fraction.

Decomposition of 4. Inorganic nitrite was measured through a chromogenic diazo coupling reaction (26). Samples were treated with 0.5 ml of 1.0% sulfanilamide in 25% concentrated HCl followed by 0.5 ml of 0.02% *N*-(naphthyl)ethylenediamine · 2HCl, and dye formation was quantitated at 530 nm. No dye formation occurred when unmodified 1 was treated with these reagents. However, as expected (27), 1 decomposed into inorganic nitrite during heating for 1.0 min at 100°C in 1.0 N NaOH. The nitrite assay thus provides a convenient and sensitive means to assay nitroheterocyclic drugs colorimetrically.

Nonenzymatic alkylation of metronidazole. Excess

iodomethane, 0.2 ml, was added to 100 mg of metronidazole dissolved in the minimal volume of dimethylformamide. After 15 h at 25°C, cyclohexane was added dropwise until crystallization was apparent. The first crop of bright yellow crystals of 3-(2-hydroxyethyl)-1,2-dimethyl-4-nitroimidazolium iodide weighed 80 mg. The electronic spectrum in 20 mM sodium acetate buffer at pH 5.0) exhibited a maximum at 278 nm ($\epsilon = 7.0 \text{ mM}^{-1} \text{ cm}^{-1}$; NMR (D_2O) 8.9 (s, 1, imidazolium), 4.9 (t, 2, $=\text{N}-\text{CH}_2-$), 4.15 (t, 2, $-\text{CH}_2-\text{OD}$), 4.1 (s, 3, $\text{CH}_3-\text{N}=\text{N}$), and 2.9 ppm (s, 3, $\text{CH}_3-\text{C}=\text{N}$).

RESULTS

We tested the drug *in vitro* as a substrate for the thiaminase secreted as an exoenzyme by the aerobic *B. thiaminolyticus* (a human colonic isolate) and for the intracellular thiaminase of the quahog clam *V. mercenaria*. Substrate activity was detected with both enzymes as metronidazole-stimulated enzymatic production of ethyl acetate-extractable radioactivity from [thiazole-2- ^{14}C]thiamine (22). At pH 6.0 and 25°C, the drug exhibited a K_m value of 3.3 mM and relative V_{max} of 0.61 when compared with aniline ($K_m = 1.2 \text{ mM}$) as a substrate for the bacterial enzyme. The drug is thus 22% as active as aniline, one of the best substrates known to be accepted by the enzyme. In the case of the molluscan enzyme, the drug ($K_m = 5.0 \text{ mM}$) was only 0.6% as active as aniline ($K_m = 0.36 \text{ mM}$).

The structures of the products of thiaminase action have been elucidated in few instances. However, we have established that the product of thiaminase action on 1 has structure 4 proposed in Scheme I. Thus, when enzymatic reaction mixtures were deproteinized and then subjected to high-performance cation-exchange chromatography, only two chromophores (I and II) with absorbance at 254 nm were found to adhere to the column at pH 4.5. The less-tightly bound chromophore (I) was identified as unreacted thiamine on the basis of its mobility, electronic spectrum, and thiochrome reaction (21). At pH 7.0, the electronic spectrum of peak II exhibits maxima at 231 and 275 nm (Fig. 1). We attribute the former to the pyrimidine moiety and the latter to the nitroimidazolium group of species 4. Metronidazole

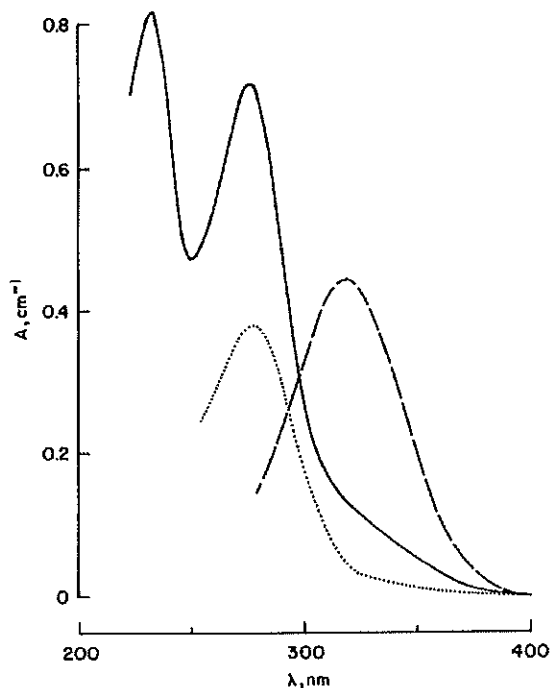


FIG. 1. Electronic spectrum of the product of thiaminase action on metronidazole. The spectrum (solid line) was recorded in 50 mM potassium phosphate buffer at pH 7.0 and 25°C after isolation of the product from the reaction mixture. The spectrum is compared with those of the neutrally charged parent drug (dashed line) and the nitroimidazolium cation obtained upon *N*-alkylation of the drug with iodomethane (dotted line).

base ($\text{p}K$ 2.7) exhibits an electronic absorption maximum at 320 nm ($\epsilon = 9310$), whereas the absorption maximum of its nitroimidazolium species in strongly acidic solution shifts to 272 nm. Similarly, we find that nonenzymatic *N*-alkylation of the drug with iodomethane shifts the absorption maximum to 278 nm ($\epsilon = 7020 \text{ M}^{-1} \text{ cm}^{-1}$) (Fig. 1). Bleaching of the absorbance at 320 nm thus confirms *N*-alkylation of the drug in the thiaminase reaction.³

³ Since a large (ca. 50 nm) hypsochromic shift in ultraviolet absorbance occurs when the drug is protonated by acid, alkylated nonenzymatically by iodomethane, or alkylated enzymatically by thiamine, it is curious that metronidazole has been claimed to be ADP-ribosylated by an NAD glycohydrolase (EC 3.2.2.5) to afford a product with an unchanged absorption maximum at 320 nm (28). That putative analog of NAD is probably not an *N,N'*-disubstituted nitroimidazolium species.

As expected from the proposed structure, formation of **4** by either the bacterial or molluscan enzyme is a reversible process. After isolation of **4** by cation-exchange chromatography, a 320-nm chromophore reforms during reincubation with thiaminase in the presence of a transparent co-substrate such as cysteine. Upon extraction into ethyl acetate, the 320-nm chromophore comigrates with authentic metronidazole during reversed-phase high-performance liquid chromatography (29).

As described under Experimental Procedures, the ^1H NMR spectrum of **4** is also consistent with the assigned structure. For instance, the imidazole proton of the neutrally charged parent drug exhibits a chemical shift of 8.2 ppm, whereas the corresponding imidazolium proton of **4** exhibits a chemical shift of 9.4 ppm. As expected by analogy with thiamine, the nitroimidazolium aromatic proton exchanges rapidly with solvent deuterons. At pD 4 and 18°C, the hydrogen exchange occurs with a half-time of less than 7 min.

When heated under alkaline conditions, metronidazole can undergo aromatic nucleophilic substitution of the nitro group. With cysteamine as nucleophile the products include imidazole thioethers and, transiently, inorganic nitrite (27). The enzyme-synthesized nitroimidazolium cation should be many orders of magnitude more susceptible to aromatic nucleophilic substitution of the nitro group than the neutrally charged drug (30). We have confirmed that **4** decomposes into inorganic nitrite under mild conditions. For instance, in 100 mM potassium phosphate buffer at pH 7.2 and 23°C, **4** releases 0.6 eq of inorganic nitrite in a first-order process with a half-time of 6.0 h. No decomposition of the parent drug occurred under those conditions.

Since alkylation by thiaminase increases the electrophilicity of metronidazole, it occurred to us that the drug might function as a suicide inactivator of the enzyme (31). However, no inactivation of the bacterial enzyme occurred during incubation for 30 min with 1.0 mM metronidazole together with 0.1 mM thiamine in 200 mM sodium

phosphate buffer at pH 5.8 and 25°C. Several other weak bases potentially alkylated to afford highly electrophilic cationic species also failed to inactivate the thiaminase under those conditions. Those compounds include pyrazinamide, 5-methylisoxazole (possibly convertible to an analog of Woodward's reagent K), 2-fluoropyridine, 2-chloropyridine, 3-(trifluoromethyl)pyridine, and 2-methoxypyridine (potentially alkylated to afford a methylating agent). Though no inactivation of the thiaminase was observed, this strategy may prove feasible for the suicide inactivation of other enzymes which alkylate heterocyclic weak bases.

When tested *in vitro* against the thiamine pyrophosphokinase isolated from baker's yeast, **4** exhibited competitive inhibition with respect to thiamine. The apparent K_i value of 80 μM for **4** equaled the apparent K_m value for thiamine. The metronidazole-derived analog is thus equipotent to the classic thiamine antagonist pyrithiamine as a competitive inhibitor of this enzyme which normally converts the vitamin to the coenzyme form (23). Pyrithiamine probably acts *in vivo* primarily through its competitive inhibition of the kinase (32).

We have not determined whether **4** is also a competitive substrate of the pyrophosphokinase. However, the coccidiostat amprolium is an effective veterinary B_1 antagonist even though it lacks a hydroxyethyl functionality (32). Thus, pyrophosphorylation is not required for vitamin B_1 antagonism.

DISCUSSION

Our results establish that, despite its poor basicity, metronidazole is sufficiently nucleophilic to be enzymatically incorporated into a thiamine analog, and the analog can inhibit pyrophosphorylation of thiamine. Our hypothesis that side effects of the drug are secondary to interference with thiamine metabolism is therefore viable. Further tests of this hypothesis will require experiments *in vivo*.

The experiments described here also

suggest other mechanisms through which metronidazole could exert side effects. Alkylation of the drug by thiaminase or, for that matter, other enzymes possibly including NAD glycohydrolase (28) and non-specific *N*-methyltransferase (33) greatly enhances the electrophilicity of the nitroimidazole. Upon *N*-alkylation the drug could therefore covalently modify nucleophilic cellular constituents. The activation observed with thiaminase action on metronidazole is an example of an established phenomenon whereby quaternization of heterocyclic nitrogen greatly enhances the electrophilicity of the parent heterocycle. For instance, susceptibility of 2-chloropyridine to nucleophilic substitution of the halogen is enhanced by a factor of 3×10^8 upon *N*-alkylation (30). Similarly, 2-methoxypyridine can be alkylated to afford a powerful methylating agent (34), and *N*-alkylation of isoxazoles can generate reactive ketenimines in aqueous solution (35). This strategy of dramatically augmenting the electrophilicity of heterocyclic weak bases through enzymatic quaternization may prove useful in the deliberate design of selectively cytotoxic agents and may pertain to adverse effects of heterocyclic drugs in addition to metronidazole.

Our observation that *N*-alkylation of metronidazole augments its electrophilicity indicates that, for much the same reason that quaternization of nicotinamide permits NAD to carry electrons, alkylation ought to also enhance the reduction potential of the nitroimidazole. Since reduction to chemically reactive products is probably the mechanism of anaerobic antimicrobial action of the drug (36, 37), it is plausible that slow reduction by mammalian enzymes (38, 39) is involved in some adverse effects of metronidazole. Enzymatic quaternization ought to increase the electron affinity of nitroheterocycles and may thus facilitate deleterious nitroreduction reactions within aerobic mammalian cells.

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