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Synthesis of 1-(2-hydroxyethyl)-2-methyl-5-aminoimidazole: a ring-intact reduction product of metronidazole

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Metronidazole, 1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole, is an antimicrobial drug with an unusual selectivity for anaerobic microorganisms. Its clinical usefulness encompasses such protozoal diseases as amoebiasis, giardiasis and trichomonal vaginitis [1]. The drug is also particularly effective in the treatment of anaerobic bacterial infections of the abdomen and pelvis as well as a number of post-operative surgical infections [1].

Despite over 20 years of clinical use, very little is known about the mechanism of microbicidal action of this compound. However, it is generally believed that cellular death is effected by some species derived from the reduction of the 5-nitro group. Edwards and coworkers [2] have presented evidence which suggests that this is brought about by inducing strand breakage in the DNA of susceptible bacteria. The requirement for reduction of the nitro group is also believed to explain the selectivity of the compound for anaerobic microorganisms and a few capnophilic bacteria. These organisms possess enzyme systems that are capable of operating at the highly negative redox potentials that are required for the reduction of metronidazole ($E_7^1 = -0.486$ mV)* [3, 4]. Nevertheless, the nature of the reduced species which is actually the agent responsible for causing cellular death is unknown. *In vitro* studies have pointed to both a four- and six-electron reduction process [5, 6]. This has led to speculation that either an amine (six-electron reduction product) or a hydroxylamine (four-electron reduction product) is the biocidal agent. Both of these classes of compounds include members that are known to be mutagens and carcinogens. They also have been shown to interact with, and cause disruption of, DNA [7, 8]. On the other hand, the amine and/or the hydroxylamine may be metabolic end-products, with the actual cytotoxic species being produced earlier in the reductive process; one possibility that has been suggested is the metronidazole radical-anion which is the product derived from a one-electron transfer to the 5-nitro group [9].

Although Goldman and coworkers [10, 11] have isolated acetamide and *N*-(2-hydroxyethyl)-oxamic acid from the urine of rats which had been given metronidazole and have obtained similar fragmentation products from the reduction of metronidazole by xanthine oxidase [12], no ring-intact reduced metabolite of metronidazole has ever been isolated. Goldman suggests that the above products arise from the hydrolytic cleavage of some reduced form of the drug. Such a process had been advanced previously to explain the inability to isolate a ring-intact reduction product, the known reactivity of 5-aminoimidazole being cited as precedent [5, 13].

This paper describes the synthesis and some of the properties of one of the postulated reduced metabolites of metronidazole, namely 1-(2-hydroxyethyl)-2-methyl-5-aminoimidazole, the six-electron reduction product.

Materials and methods

Metronidazole and 1-(2-hydroxyethyl)-2-methylimidazole were obtained through the courtesy of May & Baker Ltd., Dagenham, Essex, U.K. 2-Methylimidazole was obtained from the Sigma Chemical Co., St. Louis, MO. Palladium on charcoal (5%) was obtained from the Alfa-Ventron Division, Danvers, MA. Deuterium oxide (100 atom-percent D), dimethylsulfoxide-d_6 (100 atom-percent D) and tetramethylsilane were obtained from the Aldrich Chemical Co., Milwaukee, WI. All other solvents and reagents were obtained from the American Scientific Products Co., Bedford, MA, and were of the highest purity available.

Ultraviolet spectra were obtained in absolute ethanol using a Beckman 24 recording spectrophotometer. Nuclear magnetic resonance spectra were recorded on either a Brucker FT-60 or a Brucker HX-270 spectrometer, and are reported as ppm downfield from an internal standard of tetramethylsilane. Mass spectra were run on either a Nuclide 12-90-G or a V.G. micromass-12B mass spectrometer via direct insertion.

Thin-layer chromatography was done in butanol–water–acetic acid (4:5:1) utilizing Merck silica gel 60 fluorescent plates. Spots were visualized with ultraviolet light.

Hydrogenations were run on a Parr low-pressure hydrogenator at an initial hydrogen pressure of *ca.* 55 psi. Typically, 8.55 g (0.05 mole) of metronidazole and 5 g of 5% Pd/C were suspended in 250 ml of absolute ethanol and hydrogenated at the above stated initial hydrogen pressure until hydrogen uptake ceased (*ca.* 20 min). The solution was filtered to remove catalyst, and the ethanol was removed by evaporation under vacuum. The residue was then pumped on at low pressure (0.04 torr) for several hours to remove residual water and ethanol. This yielded 4.5 g of a rust-orange, somewhat gummy, solid which could be purified further by extraction with 3–50 ml portions of hot $CHCl_3$, filtration and evaporation of the solution under vacuum and continued evacuation of the residue for several hours at low pressure (*ca* 0.04 torr).

Mutagenicity assays were carried out using standard Ames [14] methodology, and bactericidal studies were conducted as previously described [15].

Results and discussion

Reduction of metronidazole proceeded rapidly with the uptake of three molar equivalents of hydrogen as would be required for the production of an amine. In this regard, the product was found to give positive Bratton–Marshall and Ehrlich tests [16, 17], results compatible with the presence of a primary aromatic amine. Ultraviolet spectra showed a single maximum (215 nm) whose position and

Table 1. Ultraviolet absorption spectra of 1-(2-hydroxyethyl)-2-methyl-5-aminoimidazole and related compounds

Compound	λ_{max} (nm)	ε
1-(2-Hydroxyethyl)-2-methyl-5-aminoimidazole	215	5,000
2-Methylimidazole	214	6,200
1-(2-Hydroxyethyl)-2-methyl-imidazole	212	6,200
Metronidazole	313	11,200
	235	3,700
	214	5,400

* E_7^1 is the one-electron redox potential in mV at pH 7 measured versus the normal hydrogen electrode.

molar absorptivity were quite similar to model imidazoles and quite different from its parent (Table 1). The absorptions attributable to the presence of the nitro-group have disappeared and only the shorter wavelength absorption remains. The spectrum is also similar to what has been reported for other alkylimidazoles [18].

NMR spectra also support the assigned structure (Table 2). The 2-methyl group appeared as a sharp singlet at 2.15 δ, distinctly upfield from its counterpart in metronidazole and was consistent with the results of Takeuchi *et al.* [19, 20], who studied the NMR spectra of a series of alkyl-substituted imidazoles, and similar in position to what Grimmett [18] has reported for the 2-methyl group of 2,4,5-trimethylimidazole (2.23 δ). The position of the proton at C-4 is worthy of comment. With respect to the C-4 proton of metronidazole, it has been shifted upfield by a full 2 ppm, and with respect to the same proton in 1-(2-hydroxyethyl)-2-methylimidazole it has been shifted 1 ppm upfield. Apparently, a 5-amino substituent shields that proton about as effectively as a 5-nitro substituent deshields it. Deuterium exchange experiments at high resolution (270 MHz) confirmed the presence of exchangeable protons at 4.25 and 5.20 δ, and these are assigned as given in Table 2. The signals at 2.15 δ and 5.90 δ integrated quite well for 3/1. The NH_2 protons at 4.20 δ and the methylene protons at 3.62 δ could not be integrated separately, but taken together did integrate for 6/1 relative to the absorption at 5.90 δ.

The mass spectrum showed an intense molecular ion peak at *M/z* 141 which was, in fact, the largest peak in the spectrum. Second in intensity to the above (*ca.* 50% of the base peak) were peaks at *M/z* 45 and 41 corresponding to $C_2H_5O^+$ and $C_2H_3N^+$ ions respectively. These probably resulted from loss of the hydroxyethyl group (45) followed by ring fission to produce acetonitrile (41), and the remaining fragment, $C_2H_3N_2$, did appear as a low-intensity peak at *M/z* 55. Low-intensity peaks were also observed at *M/z* 110 and 96, corresponding to loss of —CH_2OH and the total loss of the side-chain respectively. The observed spectrum was rather similar to that found for 1-(2-hydroxyethyl)-2-methylimidazole. The only major differences were that in this case loss of —CH_2OH produced the most intense peak and the molecular ion was 50% of that. Ring fragmentation was also considerably less than was found for the amine.

Thin-layer chromatography showed only two spots, one corresponding to the reduction product, the other being a small amount (5–10%) of unreduced metronidazole. Examination of the NMR and mass spectra revealed no significant impurities beyond what was found via thin-layer chromatography.

Preliminary biological testing indicates that the compound is not mutagenic to *Salmonella typhimurium* TA100, with or without the presence of a S-9 liver microsomal preparation. This fact is of interest since metronidazole is mutagenic to a nitroreductase *plus* Salmonella strain and has been suspected of being carcinogenic [1]. Preliminary studies utilizing *Bacteroides fragilis* also suggest that the amine is not bactericidal or is at least much less so than metronidazole [minimum inhibitory concentration (MIC) 256 μg/ml vs MIC of 2 μg/ml for metronidazole]. It is possible that our failure to observe mutagenicity and cytotoxicity was the result of a rapid hydrolytic reaction leading to destruction of the amine. We do not believe this to be so. The compound could be isolated from the water produced in the reduction, and a water solution of the amine showed no changes in its u.v. spectrum over 8 hr. Prolonged exposure to water (2–3 weeks) yields a product which appears to result from the addition of one molar equivalent of water. The compound has not been characterized further, but it does not appear to be any of the fragmentation products reported by Goldman and coworkers [10–12]. In pH 4.7 acetate buffer, the only difference in u.v. spectrum from that of a water solution over the same period of time was an instantaneous 3-fold increase in the molar absorptivity. In pH 7.4 phosphate buffer, which was kept at 37° for 72 hr, there did appear a small shoulder at 250 nm. In a similar experiment, in ethanol–H_2O (1 : 1) no change was observed in the u.v. spectrum over the same period of time.

While the Ames test requires *ca.* 2 days to complete, the actual mutagenic event probably occurs within 2 hr after the test compound is added to the plates, since they contain only enough histidine for two or three cycles of DNA replication.

Finally, data from our laboratory [15] indicate that metronidazole is lethal to *B. fragilis* in under 2 hr. Thus, it appears that the amine is neither the biocidal nor the mutagenic agent derived from the reduction of metronidazole. This also implies that the hydroxylamine, if formed, would not be mutagenic, since the usual pathway for the activation of an amine is via conversion to the hydroxylamine [8]. And, if one accepts the view that the mutagenic agent and the biocidal species are one and the same, then the hydroxylamine is also not the compound that causes cellular death. It then appears necessary to give serious consideration to the possibility that the cytotoxic agent derived from reduction of the drug is a species produced rather early in the reduction process, and that the amine and/or hydroxylamine are, in fact, metabolic end points, if, indeed, they are actual *in vivo* products.

In conclusion, this paper describes the synthesis and some of the properties of 1-(2-hydroxyethyl)-2-methyl-5-aminoimidazole, one of the postulated reduction products of metronidazole.

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Table 2. Chemical shifts (ppm) for 1-(2-hydroxyethyl)-2-methyl-5-aminoimidazole and related model compounds*

Compound	—CH_3	H_4	H_5	—OH	—NH_2	—CH_2CH_2—
Metronidazole	2.45	7.91		4.95		3.70, 4.31
2-Methylimidazole	2.32	6.85	6.85			
1-(2-Hydroxyethyl)-2-methylimidazole	2.25	6.90	6.60	4.12		3.55, 3.86
1-(2-Hydroxyethyl)-2-methyl-5-aminoimidazole	2.15	5.90		5.20	4.20	3.62

* Assignments for protons at carbons four and five are made by analogy to what has been reported previously for other alkyl substituted imidazoles [18–20].

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Membrane alterations associated with progressive adriamycin resistance

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The anthracycline antibiotic adriamycin is effective in the treatment of a broad spectrum of human tumors [1]. This drug inhibits both DNA and RNA syntheses [2–5], presumably by intercalating between adjacent base pairs of native DNA [6–8]. Other drug interactions may also contribute to toxicity, e.g. with membrane components [9–11], tubulin [12], and electron transport processes [13].

Several anthracycline-resistant variants have been obtained *in vitro* and *in vivo* [14–16]. These resistant cells exhibit a decreased ability to accumulate the anthracyclines as compared with parental, drug-sensitive cells; this may be related to an apparent enhanced energy-dependent drug efflux [17–19] which could limit the cytoplasmic drug level to sublethal concentrations.

Many studies on anthracycline resistance have employed cell lines that are resistant to relatively high drug concentrations. Drug resistance is usually not characterized in cell lines selected for resistance to clinically relevant drug levels. A particular resistance mechanism may become more effective with increasing drug resistance; alternatively, cells may adapt to higher drug levels by multiple mechanisms.

To address this question we isolated, from a metastatic murine tumor line, a series of variants which exhibit increasing resistance to adriamycin. The parental tumor line (MDAY-K2), originally described by Kerbel *et al.* [20–22], rapidly metastasizes to most organs of the mouse following intradermal or subcutaneous injections. The present study describes isolation of adriamycin-resistant variants of MDAY-K2 and effects of increasing drug resistance on transport and other membrane-related cell properties.

[^{14}C]Daunorubicin (30 mCi/mmole), obtained from the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, was used as a marker for anthracycline transport. Growth media and newborn calf serum were purchased from GIBCO, Grand Island, NY; fetal calf serum was provided by Sterile Systems Inc., Logan, UT. PEG* (mol. wt 6000) and Dextran T-500 (lot 7863) were obtained from the Sigma Chemical Co., St. Louis, MO, and Pharmacia Fine Chemicals, Piscataway, NJ, respectively.

The MDAY-KD2 tumor cell line was provided by Dr. Robert Kerbel, Queen's University, Kingston, Ontario, Canada. Cultures were propagated in RPMI 1640 medium supplemented with 5% fetal calf serum, 5% newborn calf serum, 10 mM HEPES buffer, pH 7.2, 100 μg/ml penicillin and 100 μg/ml streptomycin. Cultures of drug-resistant cell lines were derived by exposing cells to a specific level of adriamycin on a biweekly schedule.

The first adriamycin-resistant variants were selected in the presence of 0.04 μg/ml adriamycin, until the generation

* Abbreviations: PEG, polyethylene glycol; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; NEM, *N*-ethylmaleimide; and DPH, diphenylhexatriene.