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REDUCTION OF NITROHETEROCYCLIC COMPOUNDS BY MAMMALIAN TISSUES *IN VIVO**

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Abstract—To determine whether nitro group reduction occurs in mammalian tissues, metronidazole (0.021, 0.064 and 10 mg/kg), misonidazole (0.015 mg/kg) and nitrofurazone (0.13 mg/kg), respectively, were administered to germfree rats. A reduced metabolite [1-(2-aminoimidazol-1-yl)-3-methoxypropan-2-ol] and two of its hydrolysis products, urea and (2-hydroxy-3-methoxypropyl)-guanidine, were found in the urines of germfree rats that received misonidazole. When nitrofurazone was administered, a reduced metabolite, 4-cyano-2-oxobutyraldehyde semicarbazone, was detected in the urines. However, acetamide and *N*-(2-hydroxyethyl)oxamic acid, fragmentation products from the reduction of metronidazole, were not found in significant concentrations in the urine when germfree rats received metronidazole. Apparently metronidazole is reduced so much more slowly than misonidazole and nitrofurazone in the tissues of germfree rats that its reductive metabolites are not detectable. This observation may be explained by the one-electron reduction potentials (E_7^1) of these drugs, that of metronidazole ($E_7^1 = -486$ mV) being lower than those of either misonidazole ($E_7^1 = -389$ mV) or nitrofurazone ($E_7^1 = -257$ mV). Under these circumstances, metronidazole reduction is not detected, either because its radical anion forms more slowly than that of the other nitroheterocyclic compounds or because its radical anion interacts more rapidly with oxygen to restore the parent compound.

Most of the biological properties of the nitroheterocyclic compounds are expressed under anaerobic conditions [1, 2] and, therefore, it has been proposed that these properties depend on nitro group reduction. In support of this hypothesis are the observations that cytotoxic [3], radiation sensitizing [4], and mutagenic [5] potencies of the nitroheterocyclic compounds correlate with their one-electron reduction potentials.

Two approaches have been taken to elucidate the biologically significant intermediates that may occur during the reduction of the nitroheterocyclic compounds. One uses electron spin resonance (ESR) techniques to detect a radical anion [6, 7]; the other seeks to characterize stable metabolites formed anaerobically during the expression of biological activity [8, 9]. Each approach has its limitations. ESR techniques are not applicable to *in vivo* conditions and thus do not permit a direct correlation between chemical and biological activities. On the other hand, characterization of the stable metabolites, while permitting correlations between chemical and biological activities, only permits inferences about the chemical structure responsible for the biological acitivty.

Nevertheless, metabolites formed under anaerobic conditions may serve as markers of the chemical events that occur during the expression of the biological activity of the nitroimidazoles. It has been found, for example, that acetamide, a stable metabolite of metronidazole, forms while metronidazole exerts its microbiocidal activity in susceptible bacteria and protozoa [9, 10]. Similarly, the formation of the amine of misonidazole occurs in tissue culture under conditions where the cytotoxic and radiation sensitizing activities of misonidazole are expressed [11]. It has also been shown that reduction of nitrofurazone by bacteria leads to the formation of the stable open-chain nitrile, 4-cyano-2-oxobutyraldehyde semicarbazone [12]. It seemed logical, therefore, to seek the formation of these metabolites as a means of determining whether reduction of their respective parent nitroheterocyclic compounds might occur in mammalian tissues under physiological conditions. Information relating to the reduction of nitroheterocyclic compounds in mammalian tissues may help to understand the undesirable effects of these compounds, e.g. their possible tumorigenicity for some rodents [13, 14] and their neurotoxicity [15], as well as some of their potential therapeutic properties such as cytotoxicity [16].

There are technical problems, however, in determining whether nitro group reduction occurs in mammalian tissues under physiological conditions. Because nitro group reduction occurs in the flora of conventional laboratory animals [17], it is not possible to use the overall metabolism of compounds in these animals as a guide to what may be occurring in their tissues. Tissue cultures and perfused organs remove the effects of the flora. However, the metab-

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olism of nitroheterocyclic compounds in these systems depends on the experimentally imposed oxygen tension [18] and thus may not provide a reliable indication of what occurs physiologically. Germfree animals, on the other hand, offer a means of studying the reductive capacity of animal tissues under physiological conditions. Therefore, we administered metronidazole, misonidazole and nitrofurazone to germfree rats and sought their respective stable reductive metabolites in the urine to determine to what extent their biologically active precursors might have been formed in mammalian tissues.

In previous studies, the fragmentation of metronidazole [19, 20] and the reduction of misonidazole [21], which occur in both anaerobic bacteria and conventional rats, was not detected in germfree rats. However, the use of high doses of metronidazole and misonidazole at low specific radioactivities in these early studies may not have been suitable to detect very small amounts of metabolites characteristic of nitro group reduction. Thus, the studies in germfree rats have been repeated with metronidazole and misonidazole of high specific radioactivities in order to increase the sensitivity of detection of nitro group reduction and imidazole ring cleavage. In addition, we have sought evidence for the reduction of the nitro group of nitrofurazone in mammalian tissue by administering radiolabeled nitrofurazone of high specific activity to germfree rats and examining the urine for its stable reduced metabolite, 4-cyano-2-oxobutyraldehyde semicarbazone.

METHODS

Materials. Crystalline metronidazole (m.p. 158–160°) and [1′,2′-$^{14}C_2$]metronidazole (11.7 mCi/mmole), radiolabeled in both carbons of the ethanol side chain, were gifts from G. D. Searle & Co. (Chicago, IL). [2-^{14}C]Metronidazole (18.6 mCi/mmole) was a gift from May & Baker, Ltd. (Dagenham, Essex, England). [2-^{14}C]Misonidazole (29 mCi/mmole) and desmethylmisonidazole were gifts from Hoffmann-LaRoche, Inc. (Nutley, NJ). [1′-^{14}C]Nitrofurazone (3.54 mCi/mmole) was a gift from the Norwich-Eaton Co. (Norwich, NY); crystalline nitrofurazone was from Pfaltz & Bauer, Inc. (Flushing, NY).

Radiolabeled metronidazole, misonidazole and nitrofurazone were purified prior to use by cation exchange chromatography on an AG 50W-X4 column [19]. When this column was developed with 30 ml of water followed by 50 ml of 1 N NH_4OH, misonidazole and metronidazole were eluted at 24–56 ml and 60–68 ml respectively. Nitrofurazone was eluted at 90–108 ml when the column was further developed with 50 ml of 4 N NH_4OH. [1-^{14}C]Acetamide (1.0 mCi/mmole) was purchased from the California Bionuclear Corp. (Sun Valley, CA). [^{14}C]Urea (6.82 mCi/mmole) and [1,2-^{14}C]ethanolamine (3.9 mCi/mmole) were purchased from the New England Nuclear Corp. (Boston, MA). The sodium salt and the lactone of *N*-(2-hydroxyethyl)oxamic acid (HOA) had been synthesized previously in this laboratory [19]. 1-(2-Aminoimidazol-1-yl)-3-methoxypropan-2-ol (AIM) was synthesized by reduction of misonidazole [21]. (2-Hydroxy-3-methoxypropyl)-guanidine (G), which is a hydrolysis product of AIM [21], was obtained from the bacterial reduction of misonidazole as described previously [11]. 4-Cyano-2-oxobutyraldehyde semicarbazone (C) was synthesized by allowing the unstable amino derivative, formed by catalytic hydrogenation, to remain at pH 9 overnight [22]. All other chemicals were purchased from the Aldrich Chemical Co. (Metuchen, NJ) or the Fisher Scientific Co. (Boston, MA), unless otherwise specified.

Metabolism. Male germfree and conventional rats of the Sprague–Dawley strain (weighing approximately 250–500 g) were purchased from the Charles River Breeding Laboratories, Wilmington, MA, and were permitted Charles River rat chow *ad lib.* Metabolism studies with rats were conducted with radiolabeled drugs as described previously [23]. Urine was analyzed for the appropriate reduced metabolites.

Analyses of metabolites. To isolate radiolabeled HOA, urine from rats which had received [1′,2′-$^{14}C_2$]metronidazole was collected in 2 ml fractions in the eluate from an AG 50W-X4 column [19] and fractions 4 to 7 were then chromatographed on an AG 1-X4 column [19]; these fractions of the eluate, shown previously to contain HOA, were pooled, reduced in volume, and analyzed by thin-layer chromatography (TLC) and high pressure liquid chromatography (HPLC) [19]. Alternatively, the material from the AG 1-X4 columns was hydrolyzed with 2 N HCl for 1 hr at 122° to release radiolabeled ethanolamine whose presence was confirmed by TLC or cation exchange chromatography and assayed by liquid scintillation photometry.

To isolate radiolabeled acetamide, urine from rats that had received [2-^{14}C]metronidazole was chromatographed on an AG 50W-X4 column [19], and fractions 12 to 18 of the eluate were further fractionated by TLC on mylar sheets and assayed by liquid scintillation photometry [20].

Metabolites of misonidazole were also separated by chromatography on an AG 50W-X4 column [21]. Fractions 30 to 34, which contained AIM and urea, and fractions 50 to 55, which contained metabolite G, were then reduced in volume and the metabolites quantified by HPLC as previously described [21].

Radiolabeled C was assayed in urine by HPLC using a Whatman (Clifton, NJ) C-18 reverse phase Partisil ODS-3 column eluted at 2 ml/min with 10% methanol in water. C, with a retention time of 5.8 min under these conditions, was quantified by the radioactivity recovered in the eluate collected at 15-sec intervals between 4.5 and 6.5 min.

To determine whether the small amounts of various metabolites found in urine might be due to spontaneous hydrolysis, radiolabeled drugs (final concentrations: [1′,2′-$^{14}C_2$]metronidazole, 4 nmoles/ml; [2-^{14}C]metronidazole, 3 nmoles/ml; [2-^{14}C]misonidazole, 2 nmoles/ml; and [1′-^{14}C]nitrofurazone, 14 nmoles/ml) were added to urine obtained from either conventional or germfree rats. For germfree rats, the drugs were incubated aerobically in recently collected sterile urine at room temperature. For conventional rats, drugs were added to urine that had been collected recently in

Table 1. Metabolites of metronidazole in the urines of germfree rats*

Metabolite	Metronidazole dose (mg/kg)	Metabolite recovered† Radiolabel (%)	nmoles
Acetamide‡	0.021	0.63 (0.6, 0.7)	0.37 (0.35, 0.41)
	10.0	1 (0.1, 1.9)	290 (116, 551)
HOA§	0.064	0.17 (0.1, 0.2)	0.16 (0.09, 0.19)
	10.0	0.17 (0.1, 0.3)	25 (15, 44)
Ethanolamine‡	0.064	0.33 (0.2, 0.4)	0.31 (0.19, 0.38)
	10.0	0.17 (0.1, 0.2)	25 (15, 29)

* Three rats were each administered 1.1 μCi of metronidazole by gavage.
† Values reported are the mean with the range in parentheses.
‡ As determined by cation exchange chromatography [19].
§ As determined by thin-layer chromatography after anion exchange chromatography.

test tubes containing 1 ml toluene. Toluene was added to prevent growth of bacteria that may have entered the urine from feces. In the absence of toluene, such urines were found to reduce nitrofurazone, even in the presence of oxygen. Urines (pH approximately 6.5), collected so as to avoid bacterial growth, were incubated aerobically for 24 hr at room temperature with each of the nitroheterocyclic compounds and then analyzed for their respective anaerobic metabolites.

RESULTS

When metronidazole was administered to germfree or conventional rats, at least 90% of the urinary recovery of radiolabel appeared in the first 24 hr. Table 1 contains results of experiments in germfree rats which indicate that at low doses (10 mg/kg or less) small amounts of radiolabeled acetamide, HOA and ethanolamine were detected in the urine. The significance of these small amounts of metabolites in the urine of germfree rats was explored further by examining metabolites formed when radiolabeled metronidazole was incubated in the sterile urine obtained from germfree rats as described in Methods. These *in vitro* incubations yielded the same small amounts of acetamide, HOA and ethanolamine that had been detected *in vivo*. Similar small amounts of these metabolites were found when metronidazole was incubated *in vitro* with conventional rat urine. Therefore, the fragmentation products of metronidazole found in the urines of germfree rats could be attributed to the reaction of metronidazole in the urine and did not implicate the tissues of the germfree rats as a source of metronidazole reduction.

The finding of small amounts of metabolites derived from the fragmentation of metronidazole in the urine appears to contradict the results of earlier studies which failed to detect these metabolites in the excreta of germfree rats [19, 20]. In the earlier experiments, however, metronidazole of much lower specific radioactivity was administered at a dose of 200 mg/kg. Under these conditions the minimal amounts of acetamide and HOA detectable would be 400 and 200 nmoles, respectively, amounts considerably greater than those found in these experiments. Thus, the small amount of metronidazole reduction indicated by the metabolites found in the urine in these experiments would have escaped detection in the earlier studies. The role of the flora in the *in vivo* reduction of metronidazole [19, 20] was confirmed by administering a 0.021 mg/kg dose of [2-^{14}C]metronidazole to conventional rats and finding that the recovery of acetamide averaged 3.0% (range in three animals 2.3 to 3.8), a conversion significantly higher than that found either in the germfree rat or as the result of incubating metronidazole with rat urine.

Table 2. Metabolites of misonidazole and nitrofurazone in the urines of germfree and conventional rats

Metabolite	Radiolabel recovered (%) Germfree	Conventional	Metabolite recovered (nmoles) Germfree	Conventional
Misonidazole*				
AIM	0.5, 0.5	6.0 (2.5, 10.4)†	0.2, 0.2	2.3 (0.9, 3.9)†
Urea	2.8, 3.1	3.0 (2.6, 3.7)	1.1, 1.2	1.1 (1.0, 1.4)
G	0.8, 0.4	0.9 (0.7, 1.2)	0.3, 0.2	0.3 (0.3, 0.5)
Nitrofurazone‡				
C	0.8 (0.6, 0.9)†	1.5 (0.7, 2.3)	37 (28, 48)†	68 (31, 107)

* Two germfree and three conventional rats were each administered 1.1 μCi of [2-^{14}C]misonidazole (0.015 mg/kg) by gavage, and the metabolites were quantified as described in Methods.
† Values reported are the mean with the range in parentheses.
‡ Four germfree and three conventional rats were each administered 1.2 μCi of [1-^{14}C]nitrofurazone (0.13 mg/kg) by gavage, and metabolite C was quantified.

Small amounts of the reduced metabolites of misonidazole appeared in the urines of germfree rats (Table 2). These metabolites could not be attributed simply to an interaction with germfree urine because neither radioactive AIM nor urea or G was detected when misonidazole was incubated with urine obtained from either germfree or conventional rats. In the conventional rats, the yield of AIM was approximately ten times greater than that found in the germfree rats, although no difference was detected in the recoveries of the two other metabolites. It appears, therefore, that misonidazole reduction occurs in the tissues of the germfree rats as well as in the intestinal microflora.

The apparent discrepancy between these results and those reported previously [21] can also be explained by the limits of detection imposed by the specific activity of the misonidazole used previously. At the dose of 200 mg/kg used in the earlier studies, amounts of AIM in the urine less than 20 nmoles would have escaped detection. Thus, the smallest amount of AIM that would have been detected in previous experiments with germfree rats was approximately 100-fold greater than the amounts detected in these experiments.

The stable reductive metabolite of nitrofurazone, C, was also detected in the urine of germfree and conventional rats that received nitrofurazone at a dose of 0.13 mg/kg (Table 2). However, the amount of this metabolite in the urine of the conventional rat was approximately twice that of the germfree rats. When radiolabeled nitrofurazone was incubated in urines from either conventional or germfree rats, as described in Methods, C was not detected.

DISCUSSION

Our finding that the nitro groups of misonidazole and nitrofurazone, but not that of metronidazole, can be reduced by germfree rats is in accord with the mechanism of nitro group reduction of nitroheterocyclic compounds that has been proposed by Peterson *et al.* [7] and Perez-Reyes *et al.* [6]. ESR studies of the reduction of nitrofurazone [7] and metronidazole [6] suggest that the radical anion, which forms in the first step of nitro group reduction, disproportionates to yield the nitroso analog and the parent molecule. The nitroso analog may then undergo further reduction to form sequentially the hydroxylamine and amine analogs. In the presence of oxygen, however, the radical anion is not reduced further but reacts with oxygen to form superoxide and, in what has been termed a futile metabolic cycle [6, 7], regenerates the parent molecule. Presumably, the reduction of misonidazole occurs by the same mechanism.

It has been demonstrated that a radical anion, formed as the result of nitro group reduction, reacts with oxygen at a faster rate if it is formed from a compound with a lower E_7^1 value [24]. Thus, when the oxygen tension is the same, the radical anion of metronidazole ($E_7^1 = -486$ mV) reacts approximately twice as rapidly with oxygen as that of misonidazole ($E_7^1 = -389$ mV) and still more rapidly than that of nitrofurazone ($E_7^1 = -257$ mV) [24]. This prediction agrees with the finding that nitrofurazone disappears when incubated with rat liver microsomes even when oxygen is present [7]; the reduction of metronidazole under these circumstances unfortunately has not been examined [6]. Our finding of products more reduced than the radical anion of misonidazole and nitrofurazone but not of metronidazole in germfree rat urine is consistent with the selective reduction of nitroheterocyclic compounds that would be predicted in the presence of oxygen. However, it is also consistent with the rate of reduction of nitroheterocyclic compounds that has been observed *in vitro* under anaerobic conditions [25–27]. Thus, misonidazole is reduced an order of magnitude faster than metronidazole [26, 27] and an order of magnitude more slowly than nitrofurazone [26] by L-929 tissue culture cells, by *Escherichia coli* B/r and by liver microsomes as well as by a preparation of xanthine oxidase. Therefore, the detection of the reduced products of nitrofurazone and misonidazole, but not of metronidazole in germfree rat urine, is consistent with an *in vitro* reduction that occurs either aerobically or anaerobically.

It is not clear where nitro group reduction occurs in the germfree rat. The finding of a metabolite in extracts of liver but not of other tissues when mice were given misonidazole [28] suggests that the liver is the major site of reduction. That the liver is the site of nitro group reduction is further supported by the observation that mouse liver microsomes reduced nitrofurazone, misonidazole and metronidazole at relative rates of 1.0, 0.04 and 0.006 respectively [26].

A simple pharmacokinetic difference might account for the observed differences in reduction if, for example, more misonidazole and nitrofurazone than metronidazole were distributed to the site where reduction takes place. However, this possibility is unlikely since these agents are distributed fairly uniformly in mammalian tissues [29–31]. Thus, the one-electron reduction potential of a nitroheterocyclic compound seems to determine the extent to which it is reduced by mammalian tissues.

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