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COMPARATIVE MISONIDAZOLE METABOLISM IN ANAEROBIC BACTERIA AND HYPOXIC CHINESE HAMSTER LUNG FIBROBLAST (V-79-473) CELLS*

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Abstract—The metabolism of the radiation sensitizer misonidazole was similar in anaerobic cecal contents and hypoxic Chinese hamster lung fibroblasts (V-79-473). Both systems formed the amino derivative of misonidazole, [1-(2-aminoimidazol-1-yl)-3-methoxypropan-2-ol] (AIM), and urea, as well as a metabolite, (2-hydroxy-3-methoxypropyl)-guanidine (G), which has not been described previously. It appears that the nitro group of misonidazole was reduced to form AIM and that this compound was then hydrolyzed to yield either urea or G, the latter in yields of 25% (tissue culture) to 55% (cecal contents). When tested with the Ames tester strain, both G and AIM were slightly mutagenic only for strain TA 98 and then only in the presence of the system for microsomal activation.

Misonidazole is an effective radiosensitizer of hypoxic cells *in vitro* and is under clinical investigation as a radiosensitizer in the treatment of certain cancers [1]. The drug is more cytotoxic for hypoxic cells and it is only under hypoxic conditions that misonidazole is reduced to the amine, 1-(2-aminoimidazol-1-yl)-3-methoxypropan-2-ol (AIM), by both Chinese hamster ovary (CHO) cells and KHT fibrosarcoma cells [2]. It has been proposed that this reduction of the nitro group proceeds through the nitroso and hydroxylamine intermediates [3], and that intermediates such as these are the actual mediators of misonidazole activity [1].

Significant amounts of AIM are formed in the rat only in its anaerobic intestinal microflora [4]. In this paper we compare the metabolism of misonidazole by anaerobic bacteria with that by hypoxic mammalian cells in culture and describe the occurrence in both systems of fairly large yields of an additional metabolite, (2-hydroxy-3-methoxypropyl)-guanidine (G), that reflects nitro group reduction.

MATERIALS AND METHODS

Materials. Misonidazole, 1-(2-nitro-1-imidazolyl)-3-methoxy-2-propanol, and [2-^{14}C]misonidazole (29 mCi/mmole) were gifts of Hoffmann-La Roche Inc. (Nutley, NJ). [^{14}C]Urea (6.82 mCi/mmole) was purchased from the New England Nuclear Corp. (Boston, MA). Radioactive AIM (0.14 mCi/mmole) was synthesized by reducing misonidazole (100 mg) in methanol (10 ml) containing platinum (IV) as catalyst (10 mg) as described previously [4]. AIM was found to be stable when carried through the isolation procedures. Alpha modified minimum essential medium (α-MEM) was purchased from the Kansas City Biological Co. (Lenexa, KS). All other chemicals were purchased from the Fisher Scientific Co. (Boston, MA) unless otherwise specified.

Incubation conditions for cecal bacteria. Procedures for the preparation and incubation of cecal contents (1:10 dilution in 0.1 M phosphate buffer, pH 7.4, to yield approximately 10^{10} bacteria) were described previously [5]. After incubation for 24 hr, the reaction mixture was centrifuged at 2000 *g* for 10 min, and the supernatant solution was retained for analysis by cation exchange chromatography.

Incubation conditions for tissue culture cells. Chinese hamster lung fibroblast cells (V-79-473) were allowed to grow to confluency as a monolayer on the bottom of a 250-ml Erlenmeyer flask (10^7 cells) in α-MEM supplemented with 10% fetal calf serum (v/v) (Flow Laboratories, Bethesda, MD). In an atmosphere of 95% air/5% CO_2, the doubling times were 9–10 hr at 37°. Hypoxia was achieved by changing the atmosphere to 95% N_2/5% CO_2 1 hr prior to the addition of radiolabeled misonidazole (0.9 μCi, 0.03 μmole which had been prepared in degassed medium) through the rubber septum that sealed the flask. After a 24-hr incubation period, during which the flask was continuously gassed with 95% N_2/5% CO_2, the medium was decanted and the cells were washed once with 5 ml of fresh medium. The medium and the wash were pooled and analyzed by cation exchange chromatography.

To quantify the radiolabel remaining in the cells, the cells were harvested using 3 ml of a 0.05% trypsin–0.02% EDTA solution in PBSA [6] prepared by the dilution of 5% trypsin/2% EDTA solution 10 times (GIBCO, Grand Island, NY) and suspended in 12 ml α-MEM; a 1-ml aliquot was taken to enumerate cells. The remaining cells were collected by centrifugation at 1000 *g* for 5 min, and the super-

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natant liquid was retained. The cells were washed by resuspending them in an additional 5 ml α-MEM and were again collected by centrifugation at 1000 *g* for 10 min. The two supernatant liquids were pooled and assayed for radioactivity by liquid scintillation photometry. Cells in the pellet were lysed with 1 ml methanol and both the pellet and the supernatant solution obtained after centrifugation at 1000 *g* for 10 min were assayed for radioactivity.

Cation exchange chromatography of reaction mixtures. A 2-ml aliquot of either the tissue culture medium or the supernatant liquid from the cecal incubation mixtures was added to an AG 50W-X4 column (20 × 1.0 cm, H^+ form, Bio-Rad Laboratories, Richmond, CA) and eluted as described previously [4]. The products of misonidazole metabolism obtained after incubation in both cecal contents and tissue culture cells are compared in Table 1. Fractions from individual peaks were pooled, reduced in volume by rotary evaporation, and subjected to further analyses by high pressure liquid chromatography (h.p.l.c.), thin-layer chromatography (t.l.c.), and descending paper chromatography.

Analysis of samples by h.p.l.c. High pressure liquid chromatography was performed with a Waters Associates (Milford, MA) liquid chromatograph, model ALC/GFC 204, on a μBondapak C_{18} column that was eluted with 20% methanol in 5 mM phosphate buffer at pH 6.8; the flow rate was 2 ml/min. The eluate was monitored by a u.v. absorbance detector, model 440, operated at 254 nm. The eluate was collected at intervals of 15 sec in liquid scintillation vials and assayed for radioactivity after the addition of 10 ml Aquasol (New England Nuclear). Misonidazole, urea and AIM, with retention times of 4.3, 2.0 and 5.3 min, respectively, were quantified on the basis of radiolabel recovered at these times. For urea, which does not absorb at 254 nm, the retention value was determined using [^{14}C]urea.

Thin-layer chromatographic methods. Ascending chromatography was performed using thin-layer cellulose plates [Eastman Chromatogram sheets (20 × 20 cm mylar), Eastman Kodak Co., Rochester, NY] with fluorescent indicator, in the solvent systems described in Table 2. Some materials were visualized by u.v. fluorescence. To determine radioactivity the chromatogram sheets were cut into 0.5 cm widths, placed in glass scintillation vials with 10 ml Aquasol, and assayed by liquid scintillation photometry.

Paper chromatographic method. Descending paper chromatography on Whatman 3 MM paper with *sec*-butyl alcohol saturated with water as the mobile phase was also used [2]. Radioactivity was determined as for t.l.c.

Other methods. Radioactivity was assayed in samples (0.1 to 0.5 ml) dissolved in 4–10 ml Aquasol (New England Nuclear) by means of a Packard Liquid Scintillation Photometer, model 3003, with [^{14}C]toluene (Packard Instrument Co., Downers Grove, IL) as an internal standard. Nuclear magnetic resonance spectra were recorded with a Varian FT-80 spectrophotometer in deuterated solvents (Merck & Co., Rahway, NJ). Mass spectra were recorded on a Hitachi spectrophotometer, model RMU 6E, following electron impact ionization of the sample from a direct inlet probe.

RESULTS

Cation exchange chromatography of incubation mixtures. The distribution of radioactivity in the eluate from the AG 50W-X4 column obtained after the incubation of either cecal contents or V-79-473 cells with [2-^{14}C]misonidazole is given in Table 1. Approximately the same small amounts of radiolabel were found in fractions 4–6 and 7–9. These minor products were not characterized. Misonidazole was not recovered after incubation with cecal contents but 55.7% of it remained after incubation with the V-79-473 cells at 37° for 24 hr (fractions 18–30 in Table 1). More radiolabel was collected in fractions 31–34 (where the column eluate turns basic) from the cecal incubation mixture (34.4%) than from that

Table 1. Radiolabel in the eluate from the AG 50W-X4 column

Fraction No.	% of Total radiolabel		% of Radiolabeled metabolites	
	Cecal contents	V-79-473 cells	Cecal contents	V-79-473 cells
4–6	1.4	2.7	1.6	9.4
7–9	9.0	1.1	10.0	3.9
18–30 (Misonidazole)	0.0	55.7		
31–34*	34.4	19.2	33.0	61.9
Urea (2.0)	15.1	7.3	16.8	25.5
U-1 (2.8)	2.8	2.2	3.1	7.7
U-2 (3.5)	6.6	4.4	7.3	15.4
Miso (4.3)	4.7	1.5		
AIM (5.3)	5.2	3.8	5.8	13.3
(Subtotal)	(34.4)	(19.2)	(33.0)	(61.9)
51–57	49.8	7.1	55.4	24.8
Total recovery	94.6	85.8	100.0	100.0

* Fractions 31–34 were found by h.p.l.c. to separate into five components. The identity of these components when known and their high pressure liquid chromatographic retention times (in min) are indicated, and the percentages of total radiolabel that they represent are given in the column inserts. Key: U-1 and U-2, unidentified metabolites; Miso, misonidazole; and AIM, 1-(2-aminoimidazol-1-yl)-3-methoxypropan-2-ol.

Table 2. Thin-layer chromatographic systems

Sample	R_f values [Propanol/NH_4OH (7:3)] Standard	Cecal contents	Tissue culture	R_f values [*n*-butanol/NH_4OH (14:3)] Standard	Cecal contents	Tissue culture
Fractions 31–34						
Unknown 1		0.05	0.05		0.03	0.03
		0.16	0.22			
Unknown 2		0.34	0.34		0.20	0.22
Unknown 3					0.43	
Urea	0.53	0.53	0.50	0.34	0.31	0.37
AIM	0.75	0.78	0.75	0.74	0.71	0.74
Miso	0.84			0.80		
Fractions 51–57		0.44	0.47		0.17	0.21

of tissue culture cells (19.2%). The major radiolabeled product of the cecal incubation mixture (49.8%) was in fractions 51–57 but these fractions contained only a small amount of radiolabel from the cell media (7.1%).

No metabolites of misonidazole were detected by h.p.l.c. or cation exchange chromatography when either cells or cecal contents were incubated under aerobic conditions. Since less than 0.15% of the radiolabel was detected in the cell lysate, after aerobic incubation, it was not analyzed further.

Characterization of (2-hydroxy-3-methoxypropyl)-guanidine (G). Fractions 51–57 obtained from the ion exchange chromatography of incubation mixtures containing either cecal contents or V-79-473 cells yielded a single radiolabeled peak with a retention time of 3.8 min on h.p.l.c. A single peak was also found by thin-layer chromatography in two systems (Table 2) and by descending paper chromatography (R_f 0.55). On this basis, the metabolite was considered to be a single substance whose identity as compound G (Fig. 1) was confirmed by n.m.r. and mass spectrometry.

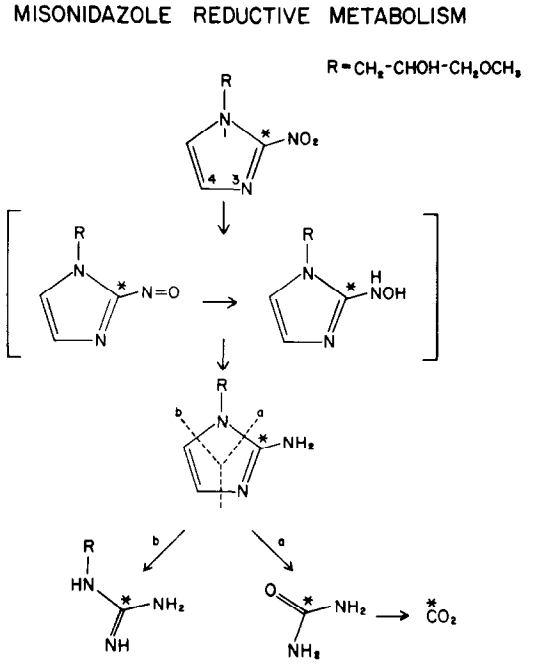

Fig. 1. Scheme for the metabolism of misonidazole by anaerobic cecal bacteria and hypoxic tissue culture cells to yield 1-(2-aminoimidazol-1-yl)-3-methoxypropan-2-ol, (2-hydroxy-3-methoxypropyl)-guanidine, and urea. The production of CO_2 from urea was demonstrated earlier in cecal contents [4]. The compounds in brackets are presumed intermediates in nitro group reduction.

The nuclear magnetic resonance spectrum (in deuterated *p*-dioxane, signal at 3.6 ppm/D_2O, 4.6 ppm) showed a multiplet at 3.9 ppm interpreted to be the methine proton of the propyl side chain, a doublet signal at 3.4 ppm and a triplet at 3.2 ppm interpreted to arise from the methylene protons at C_3 and C_1 of the propyl side chain, respectively, and a strong singlet at 3.3 ppm (between the methylene signals) interpreted to be due to the methyl group. Integration of the signals was consistent with eight protons having a ratio of 1:2:3:2. The single hydroxyl proton and all N-H protons had exchanged with D_2O.

Mass spectral analysis of the product in fractions 51–57 revealed a molecular ion at *m/e* 147. The other major ions appeared at *m/e* 132, 116, 102, 72, 58 and 43 which were consistent with the structure of G (mol. wt. 147.17).

Quantification and comparison of products. To compare our findings with those of others [2, 7, 8], we repeated their separation by descending paper chromatography using *sec*-butanol saturated with water as the mobile phase. We found that this system was capable of resolving AIM, urea and G, with respective R_f values of 0.45, 0.50, and 0.55 from

Table 3. Mutagenicity of metabolites of misonidazole in the Ames test*

Samples (μg/plate)	TA 98 $-S_9$	TA 98 $+S_9$	TA 100 $-S_9$	TA 100 $+S_9$
Control	23	37	170	170
2-Nitrofluorene (10)	4650	1500		
Misonidazole (125)	23	68	6500	5240
(1250)	135	249		
AIM (125)	23	48		
(1250)	23	69		
G (125)	23	54		
(1250)	23	76		

* Both AIM and G were tested for mutagenicity with Ames salmonella tester strains TA 98, 1537, 1538 and 100 both with and without the S9 liver microsomal activating system.

misonidazole (R_f 0.84), but that it was incapable of resolving them from each other.

Testing of metabolites for mutagenesis. Metabolites were tested for mutagenic activity by the Ames salmonella test with frame shift mutant tester strains TA 98, 1537 and 1538 and the base pair mutant tester strain TA 100 [9]. Slight mutagenic activity was detected with AIM and G only in strain TA 98, and this only when the S-9 activation system was added (Table 3). However, the revertant response was small compared to that found with 2-nitrofluorene and failed to follow a dose–response relationship.

DISCUSSION

Under the conditions we have used, it appears that similar amounts of urea and AIM are formed when misonidazole is metabolized anaerobically in either cecal contents or fibroblast cells. The formation of G was greater in cecal contents, however, indicating that there may be a preference for imidazole ring cleavage of AIM to take place according to pattern a (Fig. 1) in tissue culture and according to pattern b in cecal bacteria. The bacterial preferences for pattern b are suggested by the rather low yields of urea detected when AIM was incubated with either *Clostridium perfringens* or mixed cecal bacteria [4].

AIM and G were slightly positive as mutagens with the Ames tester strain TA 98 but only at high concentrations and with the addition of a system to provide microsomal activation. This suggests that these reduced products of misonidazole metabolism may become mutagens only after they are further metabolized to their hydroxylamine or *N*-oxide derivatives. This possible interplay between reductive and oxidative metabolism is similar to some findings by Whitmore *et al.* [10], who reported that products formed from the chemical reduction of misonidazole were more toxic to aerobic than to hypoxic Chinese hamster ovary cells. Misonidazole itself, however, is mutagenic for Ames tester strain TA 100 (a base pair tester strain) even in the absence of the microsomal system [11].

We have shown that the descending chromatographic system used previously to describe the metabolism of misonidazole in HeLa, CHO or KHT cell cultures [2, 7, 8] would not have been successful in resolving urea and G from AIM and, thus, if these metabolites were present their detection might have been obscured. Unlike previous workers [2], we were unable to find evidence for the intracellular binding of radioactive misonidazole. Perhaps the discrepancy lies in their failure to wash the cells before they were lysed.

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