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INTERACTION OF METRONIDAZOLE WITH *ESCHERICHIA COLI* DEOXYRIBONUCLEIC ACID

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Abstract—To define the characteristics of the reported binding of metronidazole to DNA, we isolated the DNA from hypoxic incubation mixtures that contained both [^{14}C]metronidazole and metronidazole-susceptible strains of *Escherichia coli*. Thus, either [2-^{14}C]metronidazole or [1′,2′-^{14}C]metronidazole was incubated with either wild-type *E. coli* (strain AB1157) or a DNA repair mutant (strain SR58) that is highly susceptible to metronidazole. Approximately 0.02% of the radiolabel in the metronidazole was found to be associated with DNA isolated from both strains of bacteria, a percentage similar to that found to be associated with DNA from mammalian sources in a variety of *in vitro* and *in vivo* experiments performed by other investigators. The bound radioactivity was not diminished, however, when a great excess of non-radiolabeled metronidazole was included in the incubation mixture, indicating that the binding we observed was probably due to impurities in the radiolabeled metronidazole. We also examined the binding to DNA of a possible surrogate for the partially reduced form of metronidazole, 1-methyl-4-phenyl-5-nitrosoimidazole (5NO), that has been described previously. The binding of the tritiated form of 5NO to DNA was also found to be undiminished by the addition of carrier 5NO (a finding which does not refute the hypothesis that 5NO may serve as a surrogate for the study of the active form of metronidazole). These studies do not exclude the binding to DNA of either metronidazole or a possible surrogate of its active functionality, but they indicate that if such binding occurs, it must be limited to very few sites on DNA and hence will be difficult to characterize.

Metronidazole and other 5-nitroimidazole compounds, used for the treatment of protozoal diseases as well as anaerobic bacteria infections [1], are thought to exert their effects by interacting with the DNA of target organisms. Thus, metronidazole is mutagenic for the Ames tester strains of *Salmonella typhimurium* [2] and is more potent for *Escherichia coli* mutants that are defective in DNA repair than for the wild type [3–5]. Metronidazole has also been reported to inhibit DNA synthesis in both cultured mouse cells and *Clostridium bifermentans* [6, 7].

Nitroreductase activity appears to be required for the activation of metronidazole. Thus, metronidazole is less mutagenic for an Ames tester strain that has diminished nitroreductase activity [2] and is less potent for strains of either *Bacteroides fragilis* [8] or *Trichomonas vaginalis* [9] with diminished nitroreductase activity. Kinetic data suggest that metronidazole is reduced by susceptible microorganisms to a partially reduced intermediate that can either react with DNA or with water [3, 10], the reaction with water resulting in the formation of such metabolites as acetamide and *N*-2-(hydroxyethyl)oxamic acid [11, 12]. These metabolites can be recovered in the excreta of conventional rats dosed with metronidazole [11, 12] but not in the excreta of germfree rats [13]. Thus, the reductive reaction of metronidazole may only occur in the flora, raising the possibility that the flora may play an obligatory role in the tumorigenicity of metronidazole for rats and mice [14].

The hypothesis that the flora are obligatory for the tumorigenicity of metronidazole might be tested by comparing metronidazole's tumorigenicity in conventional and germfree rodents. Such experiments are costly, however, because they require maintaining large numbers of animals for long periods of time. Furthermore, such comparative experiments may provide misleading results because biological differences between germfree and conventional animals might change either the rate of formation or the pharmacokinetics of the ultimate carcinogen (see, for example, Refs. 15 and 16) as well as its relationship to tumor incidence. A more direct and less costly method of investigating a possible difference in the carcinogenicity of metronidazole for germfree and conventional rats might be to measure the binding of metronidazole to DNA.

Radioactivity from metronidazole becomes associated with the DNA of mice when they are treated with radiolabeled metronidazole [17], suggesting that it might be possible to see differences in metronidazole bound to DNA in germfree and conventional rats. A number of *in vitro* experiments confirm the binding of radioactivity from either [^{14}C]- or [^{3}H]metronidazole when it is reduced in the presence of DNA [18–20]; in some experiments such binding was found to be radiation dependent [21]. Reports of the binding of metronidazole to DNA are in accord with the earlier suggestion that radiolabel from metronidazole binds to both DNA and protein of *T. vaginalis* [22] and is consistent with the reported dithionite-dependent formation of an unstable adduct between the hydroxylamino functionality of metronidazole and guanosine [23].

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In all these studies, however, the radiolabel associated with DNA represents only a very small fraction of metronidazole that is either administered to the animal or reduced in the presence of DNA. Furthermore, no difference in overall tissue binding of another 5-nitroimidazole, ronidazole, was found when this compound was administered to both germfree and conventional rats [24]. The form in which metronidazole is bound to DNA or other tissue constituents has not been determined.

Characterization of the apparent binding of metronidazole to DNA may help us to understand the possible risks [1] of this widely used drug. In addition, the binding of metronidazole to DNA might provide a way to investigate the significance of the reductive metabolism of metronidazole by the anaerobic flora. If the binding of metronidazole to DNA were characterized, the role of the flora in its activation might be inferred by comparing the binding of metronidazole to DNA in germfree and conventional animals.

We now report an investigation of the possible binding of metronidazole to the DNA of wild-type *E. coli* and to a more susceptible mutant of *E. coli* that is defective in DNA repair. To detect binding to DNA that might occur with only a portion of the metronidazole molecule, we used metronidazole radiolabeled at two different positions. Our results indicate that most binding can be attributed to impurities in the radiolabeled metronidazole and that if specific binding occurs, it appears to be quantitatively too small to characterize. A similar conclusion was reached using radiolabeled 5NO.

MATERIALS AND METHODS

Chemicals. Phenol, chloroform, isoamyl alcohol, EDTA, sodium chloride, and HPLC grade solvents were purchased from Fisher Scientific (Medford, MA). Tris salts, lysozyme, sodium docecyl sulfate, 8-hydroxyquinoline, and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO); proteinase K, ribonuclease A, and ribonuclease T1 were purchased from Boehringer Mannheim (Indianapolis, IN). Metronidazole was a gift from G. D. Searle & Co. (Chicago, IL). Radiolabeled [2-^{14}C]metronidazole (sp. act. 18.1 mCi/mmol), a gift from May & Baker Ltd. (Dagenham, U.K.), had a purity >98%, having a single additional peak consisting of 0–2% of the radiolabel, as determined by reverse phase HPLC using a Waters Associates liquid chromatograph (Milford, MA) equipped with a C18 column, 25 cm × 4.65 mm, 5 μm particle size (Supelco Inc., Bellefonte, PA). Isocratic elution, at a flow rate of 1 mL/min was with a mobile phase of 5 mM potassium phosphate, pH 4.0, that contained 20% methanol. [1′,2′-^{14}C]Metronidazole (sp. act. 12.6 mCi/mmol), a gift from G. D. Searle & Co., was found to have a purity of <95% and was therefore purified to approximately 98% by HPLC, as described above, immediately before use.

Unlabeled 1-methyl-4-phenyl-5-nitrosoimidazole was synthesized as previously described [25]. 1-[^{3}H-methyl]4-Phenyl-5-nitrosoimidazole ([^{3}H]5NO, sp. act. 7.7 Ci/mmol) was synthesized using tritiated methyl iodide by Chemsyn Science Laboratories (Lenexa, KA) from 4(5)-nitroso-5(4)-phenyl-imidazole which was synthesized in our laboratory as previously described [25, 26]. An aliquot of this radiolabeled material was diluted to a specific activity of 0.5 Ci/mmol by the addition of carrier 5NO and was found to have a purity >95% by normal phase HPLC when eluted from a Diol column (25 cm × 4.6 mm, 5 μm particle size; Supelco, Inc.), with a mobile phase having equal parts of hexane and ethyl acetate. The eluting solvent was then removed by evaporation under a stream of N_2 and the residue dissolved in DMSO.

Growth of bacteria. Wild-type (strain AB1157) and a DNA repair mutant (strain SR58) of *E. coli* [3] were grown aerobically to stationary phase on a trypticase soy medium (Becton Dickinson & Co., Cockeysville, MD). Bacteria from between 1 and 1.5 L of this medium were collected by centrifugation (7700 *g* for 20 min at 4°). After washing the bacterial pellets in cold 0.9% NaCl, the bacteria were resuspended in 15 mL of fresh trypticase soy medium. The concentration of bacteria was assayed after serial dilution in sterile 0.9% NaCl by enumerating colonies that formed on trypticase soy plates (2% agar, Becton Dickinson & Co.) that had been incubated at 37° for 24–48 hr.

Incubation of metronidazole and 5NO with E. coli. Radiolabeled metronidazole of various specific activities was added to a suspension of *E. coli* that had been made anaerobic by purging with a gas (85% argon, 10% hydrogen, 5% carbon dioxide) delivered by a V.P.I. anaerobic culture system (Bellco Glass Co., Vineland, NJ). After the tubes had been purged, they were stoppered tightly and incubated for various times at 37°. Incubation of 5NO was done similarly, except that anaerobic purging was omitted.

Isolation of DNA. At the end of the incubation period, bacteria were collected by centrifugation (at 7700 *g* at 4° for 20 min) and washed twice with 3 mL of 0.9% NaCl and the bacteria were lysed by incubating them with occasional shaking in 7.7 mL of 10 mM Tris that also contained 0.15 M NaCl, 0.1 M EDTA, pH 8.0, with lysozyme (2 mg/sample) for 30 min at 37°. Sodium dodecyl sulfate was then added at a final concentration of 1.4% and the samples were incubated at 60° for an additional 10 min [27]. When they had cooled to room temperature, the samples were treated with proteinase K (0.5 mg/mL) and incubated at 37° for 30 min, after which they were extracted with an equal volume of phenol containing 8-hydroxyquinoline (1%), the phenol having been first equilibrated with 1.0 M Tris–HCl, pH 8.0, and then with 0.1 M Tris–HCl, pH 8.0 [28]. The phenol phase was then re-extracted with an equal volume of TE, pH 7.8 (10 mM Tris–HCl, pH 7.8, 1 mM EDTA, pH 8.0) [29]. The aqueous phases were pooled and extracted repeatedly with an equal volume of a mixture consisting of 50% phenol (that had previously been equilibrated as above), 48% chloroform, and 2% isoamyl alcohol (by vol.) until no precipitate remained at the solvent interface. The aqueous phase was then treated with a 10% addition (v/v) of 3.0 M sodium acetate, pH 5.2, and nucleic acids precipitated by the addition of 2 vol. of chilled

Table 1. Radiolabel from [2-^{14}C]metronidazole associated with *E. coli* SR58 DNA after incubation under various conditions

Incubation time (min)	Carrier ratio	260/280 Ratio*	DNA recovered (μg)	Specific activity (dpm/μg)
5	0	1.58	389	ND
	10	1.61	399	ND
30	0	1.58	323	ND
	10	1.60	321	ND
240	0	1.71,1.81	273,386	0.33,0.29
	10	1.53,1.57	312,327	0.30,0.26
240	0	1.64,1.72	231,314	0.28,0.28
	10	1.78,1.79	233,342	0.45,0.38
	50	1.67,1.68	318,401	0.34,0.32
240	0	1,79,1.63	420,322	0.27,0.31
	1000	1.73,1.75	883,834	0.13,0.14

Reaction mixtures contained 12.4 nmoles (5×10^5 dpm) of [2-^{14}C]metronidazole either incubated alone or with unlabeled carrier metronidazole in the indicated ratio to radiolabeled metronidazole and 2×10^{10} *E. coli* strain SR58 in 4.3 mL trypticase soy broth. Incubation was performed anaerobically at 37°. ND indicates that radiolabel was indistinguishable from background.

* A 260/280 absorbance ratio of 1.8 is considered to be pure DNA [23].

ethanol. After remaining at −20° for several hours, the nucleic acid precipitate was collected by centrifugation at 10,000 *g* for 10 min at 0° and then redissolved in 3 mL TE, pH 7.8. The solution was then treated with ribonuclease A (0.15 mg/mL) and ribonuclease T1 (75 units/mL), incubated at 37° for 30 min to remove contaminating RNA, and afterwards extracted with an equal volume of chloroform : isoamyl alcohol (24 : 1, v/v); the aqueous phases were saved and the DNA was precipitated by the addition of 10% (v/v) 3.0 M sodium acetate, pH 5.2, and ethanol as described above. DNA was then resuspended in 2 mL of TE, and the cycle of precipitation with sodium acetate and ethanol and subsequent dissolution in TE repeated until no radioactivity could be detected in the ethanol solution after the precipitated DNA had been collected by centrifugation. DNA was quantified by absorbance at 260 nm (48 μg/absorbance unit) [30] and its radioactivity assayed with a Packard TriCarb 4530 liquid scintillation counter (Packard Instrument Co., LaGrange, IL) after the samples had been dissolved in 15 mL Scintiverse LC (Fisher Scientific); counting efficiencies were determined by an external standard and corrected for background radioactivity. Radioactivity was considered significant only if greater than twice background (36 dpm for ^{14}C, 80 dpm for ^{3}H).

RESULTS

Examining the binding of radiolabeled metronidazole to the DNA of E. coli *strain SR58.* When radiolabeled [2-^{14}C]metronidazole was incubated with *E. coli* SR58 for periods up to 30 min, no binding to DNA could be detected (Table 1). Binding of radiolabel to the extent of approximately 0.02% occurred, however, when the incubation was continued for 4 hr. The radioactive material bound to DNA likely represented an impurity, however, because it was not decreased significantly when the radiolabeled metronidazole in the incubation mixture was diluted up to 50-fold by the addition of unlabeled metronidazole. Thus, as shown in Table 1, the binding of radiolabeled metronidazole to DNA was diminished only negligibly until a 1000-fold excess of unlabeled metronidazole was added, this amount causing approximately a 2-fold decrease in the binding of radiolabeled material. Such results suggest that the apparent binding of metronidazole is caused largely by a radioactive impurity, whose specific activity is only diluted to a significant extent by large amounts of the non-radiolabeled metronidazole.

The apparent binding to DNA of metronidazole radiolabeled in the side chain was also not diminished when the same experimental protocol was repeated with [1′,2′-^{14}C]metronidazole and a 6-fold excess of non-radiolabeled metronidazole was added (Table 2).

Results consistent with those above were also obtained when wild-type *E. coli* (strain AB1157) were used (Table 3) instead of the DNA repair mutant. With this *E. coli* strain a 2-fold decrease in apparent binding also occurred when metronidazole, radiolabeled in either position, was diluted 1000-fold. Thus, the binding of metronidazole radiolabeled in either the imidazole ring or the side chain was diminished by no more than 2-fold even in the presence of 1000-fold excess of non-radiolabeled metronidazole.

Since the defect in DNA repair activity of strain SR58 makes it more susceptible to metronidazole, it was of interest to compare the apparent relative binding of metronidazole to these two strains. In the 4-hr incubations with [2-^{14}C]metronidazole, binding to SR58 DNA was 0.29 ± 0.05 (SD) dpm/μg (Table 1) while that to AB1157 was essentially the same (Table 3). The 4-hr incubations with [1′,2′-^{14}C] metronidazole yielded 0.25 dpm/μg with strain SR58 and between 0.08 and 0.30 dpm/μg with strain

Table 2. Radiolabel from [1′,2′-^{14}C]metronidazole associated with *E. coli* SR58 DNA after incubation under various conditions

Incubation time (min)	Carrier ratio	260/280 Ratio*	DNA recovered (μg)	Specific activity (dpm/μg)
240	0	1.68	317	0.25
	6	1.54	383	0.25

Reaction mixtures contained 20 nmol (5.3×10^5 dpm) of [1′,2′-^{14}C]metronidazole either incubated alone or with a 6-fold excess of unlabeled carrier and 3×10^{10} *E. coli* strain SR58 in 4.3 mL trypticase soy broth. Incubation was performed anaerobically at 37°.

* A 260/280 absorbance ratio of 1.8 is considered to be pure DNA [23].

Table 3. Radiolabel from [^{14}C]metronidazole associated with *E. coli* AB1157 DNA

Radiolabel	Carrier ratio	260/280 Ratio*	DNA recovered (μg)	Specific activity (dpm/μg)
2-^{14}C	0	1.73,1.61	302,360	0.25,0.26
	1000	1.82,1.96	199,444	0.19,0.14
1′,2′-^{14}C	0	1.74	267	0.30
	1000	1.80	748	0.13
	0	1.55	557	0.08
	1000	1.82	450	ND

Reaction mixtures contained 5×10^5 dpm of either [2-^{14}C]metronidazole (12.4 nmol) or [1′,2′-^{14}C]metronidazole (17.9 nmol) incubated either alone or with unlabeled carrier metronidazole in the indicated ratio to radiolabeled metronidazole and 9×10^{10} *E. coli* strain AB1157 in 4.3 mL trypticase soy broth. Incubation for 4 hr was performed anaerobically at 37°. ND indicates that radioactivity was indistinguishable from background.

* A 260/280 absorbance ratio of 1.8 is considered to be pure DNA [23].

AB1157. Thus, there was no indication that the apparent binding to DNA was different for the more metronidazole-susceptible repair mutant and the wild type.

Binding of [^{3}H]*5NO to DNA* E. coli *AB1157 and SR58.* It was also of interest to determine whether 5NO, a compound with the properties expected for the reactive form of a 5-nitroimidazole [25], might interact with DNA. As with metronidazole itself, a small amount of binding of [^{3}H]5NO was found to be associated with DNA of both the wild type and the DNA repair mutant. The small amount of binding of radiolabeled 5NO to DNA was also not diminished, however, when the incubation with *E. coli* was carried out in the presence of a 100-fold excess of unlabeled 5NO (Table 4). As with metronidazole, the amount of 5NO bound to DNA was low, being only approximately 0.01 to 0.03% of that added to the incubation mixture. The higher specific activity of the DNA isolated after treatment with 5NO probably reflects both the higher specific activity of the starting material and the different nature of impurities from this compound.

That the binding of 5NO to DNA also is not decreased by the addition of non-radioactive carrier is in accord with the proposal that 5NO can be considered a surrogate for the active form of metronidazole. Our studies therefore do not refute the hypothesis that 5NO may serve as a surrogate for the biologically active form of such drugs as metronidazole; they only indicate that the nature of the interaction of 5NO with DNA cannot be defined by this experimental approach.

DISCUSSION

Our studies indicate that the small fraction of radiolabeled metronidazole associated with *E. coli* DNA is not diminished to any significant extent by the addition of a considerable excess of non-radiolabeled metronidazole. Such results have at least two possible explanations. One is that our procedures for extracting DNA may have broken biologically significant covalent bonds between DNA and either metronidazole or one of its metabolites. The other is that the radioactivity associated with DNA is simply derived from an impurity in the radiolabeled metronidazole. The solvent partitioning method we used to extract DNA for the detection of adducts is essentially the same as that used by other laboratories that have successfully isolated adducts of carcinogens [30–32]. It is possible, however, that an adduct(s) derived from metronidazole is less stable than those derived from more potent carcinogens and may not have withstood the conditions used to rupture bacteria before extracting DNA [27]. Indeed it has been claimed that an adduct formed *in vitro* between metronidazole and guanosine is quite unstable [23], suggesting that a biologically significant adduct formed from metronidazole might be too unstable to withstand the conditions under which DNA is usually extracted. Nevertheless, our

Table 4. Radiolabel from [^{3}H]5NO associated with *E. coli* DNA

E. coli strain	Carrier ratio	260/280 Ratio*	DNA recovered (μg)	Specific activity (dpm/μg)
SR58	0	1.68,1.91	449,552	2.07,1.89
	100	1.66,1.80	303,481	2.27,1.81
SR58	0	1.60,1.53	325,314	3.54,4.63
	100	1.59,1.43	373,219	3.75,6.38
AB1157	0	1.45	360	1.88
	100	1.62	479	1.64
	0	2.25	181	2.12
	100	2.11	195	1.89

Reaction mixtures contained 5 nmol (5.8×10^6 dpm) [^{3}H]5NO incubated alone or with a 100-fold excess of unlabeled carrier and 9×10^{10} *E. coli* strain AB1157, or 4×10^{10} *E. coli* SR58 strain in 3.3 mL trypticase soy broth. Incubation was for 4 hr at 37°.

* A 260/280 absorbance ratio of 1.8 is considered to be pure DNA [23].

isotope dilution experiments clearly indicate that the radiolabel found to be associated with DNA can be attributed mainly to a small amount of an impurity in the radiolabeled metronidazole. This explanation is consistent with the purity of the metronidazole used (98%) and the observation that only approximately 0.02% of the radiolabel originally in metronidazole is isolated with DNA.

We cannot exclude the possibility, however, that, a small portion of the radioactivity found to be associated with DNA is actually derived either from metronidazole or from one of its biologically significant metabolites. Such biologically insignificant radioactivity may be small in comparison with that from radioactive impurities in the metronidazole. Our data indicate an association with DNA that is equivalent to one molecule of metronidazole per approximately 200,000 base pairs. The isotope dilution experiments indicate, however, that at least 90% of this radioactivity can be attributed to an impurity in the radioactive metronidazole. Thus, binding of metronidazole, or a biologically significant metabolite, can be no greater than approximately one molecule per million base pairs.

The biological significance of the apparent binding of radiolabeled metronidazole to DNA may be questioned on other grounds. Thus, such binding is not observed until at least 30 min after the bacteria have been in contact with metronidazole, a time when the bactericidal activity of metronidazole toward *E. coli* strain SR58 is already quite pronounced [33]. There is, therefore, a dissociation between the biological activity of metronidazole and the kinetics of its apparent binding to DNA. Furthermore, the apparent binding of metronidazole to DNA might be expected to be greater in strain SR58 than in strain AB1157, since the former has a defect in its ability to repair DNA that increases its susceptibility to metronidazole. Thus, although 2-nitropyrene, a carcinogen containing a nitro group, has been shown to form a covalent adduct to guanosine residues in DNA [31] and LaRusso *et al.* have evidence that metronidazole reduced *in vitro* also forms an adduct with guanosine residues of DNA [19], such adducts do not appear to form to any appreciable extent between metronidazole and the DNA of susceptible bacteria. It is possible, therefore, that metronidazole does not bind to DNA, but rather interferes in some other way with DNA function, for example, by causing strand breaks as was suggested [34–36], but not confirmed [18], by *in vitro* studies.

It is uncertain whether our results in bacteria can justifiably be interpreted to cast doubt on previous studies suggesting that metronidazole binds to DNA, but certainly such studies [17–22] should be reexamined with the results of the present studies in mind. The extent of the possible biologically significant binding of metronidazole to bacterial DNA suggests, however, that the previously described binding of metronidazole to mammalian DNA may have been greatly overestimated and that such measurements may not be useful in comparing the activation of metronidazole in germfree and conventional rats or mice.

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