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THIOL-MEDIATED INCORPORATION OF RADIOLABEL FROM 1-[^{14}C]-METHYL-4-PHENYL-5-NITROSOIMIDAZOLE INTO DNA

A MODEL FOR THE BIOLOGICAL ACTIVITY OF 5-NITROIMIDAZOLES

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Abstract—1-Methyl-4-phenyl-5-nitrosoimidazole (5NO), which has properties consistent with the biologically active form of a 5-nitroimidazole, was radiolabeled (1-[^{14}C]-methyl) and shown to bind to DNA, but at a rate too slow to account for its bactericidal effect. In the presence of physiological intracellular concentrations of such thiols as glutathione, however, binding was enhanced by 2–3 orders of magnitude, which is quantitatively sufficient to account for the bactericidal effect of 5NO. That 5NO binding was greater for poly[d(G-C)·d(G-C)] than for poly[d(A-T)·d(A-T)] suggests that the reactive species binds to nucleophilic bases on DNA, a suggestion which is also supported by our finding of a thiol-dependent reaction to form an adduct between 5NO and aniline.

The model 5-nitroimidazole, 1-methyl-4-phenyl-5-nitroimidazole ($5NO_2$)†, has bactericidal, cytotoxic and mutagenic activity similar to that of such therapeutically active 5-nitroimidazole drugs as metronidazole [1, 2]. The corresponding 5-nitrosoimidazole, 1-methyl-4-phenyl-5-nitrosoimidazole (5NO), is of particular interest because it is considerably more potent and much more labile in the presence of cells than the 5-nitroimidazole [1, 2]. The nitrosoimidazole, therefore, has the characteristics expected of the reduced functionality postulated to mediate the biological activity of the 5-nitroimidazoles [3, 4]. Further experimental work clarifying the mechanism of action of 5-nitroimidazoles is necessary to decide whether 5NO is a biologically active form of $5NO_2$, or merely more readily converted to such a form than the nitroimidazole itself.

Although DNA is apparently the target for the biologically active form of the 5-nitroimidazoles [3–5], we now find no indication that 5NO reacts with DNA when the two are incubated together in phosphate buffer. Further reduction of the nitrosoimidazole may be required, but another possibility is that an interaction with another compound may be required to activate the nitrosoimidazole. Nitrosoimidazoles react rapidly with thiol compounds [1], a finding which may account for the rapid decomposition of nitrosoimidazoles in the presence of eucaryotic or procaryotic cells [1, 2].

Thiols also react rapidly with such other nitroso-containing compounds as the nitrosoarenes [6–8]. The mechanism postulated for that reaction suggests that a thiol compound may interact with 5NO *in vivo* to form a reactive species, which in this case either quickly decomposes by hydrolysis or reacts with DNA to form a covalent bond.

Therefore, we examined the interaction of 1-[^{14}C]-methyl-4-phenyl-5-nitrosoimidazole (1-[^{14}C]-5NO) with both *Escherichia coli* lambda-phage DNA and synthetic double-stranded polydeoxynucleotides and found evidence for a quantitatively significant reaction only in the presence of such thiols as cysteine and glutathione. The results of these studies and the possible mechanism responsible for them are the subject of this paper.

MATERIALS AND METHODS

Ultraviolet spectral data were obtained using a Hewlett–Packard (Palo Alto, CA) model 8451A diode-array spectrophotometer, and mass spectra (MS) with a VG Analytical ZAB-SE mass spectrometer using FAB or ammonia chemical ionization. Radioactivity was assayed with a Packard TriCarb 4530 liquid scintillation spectrophotometer (Packard Instrument Co., LaGrange, IL) in samples prepared by dissolving them in 15 ml Hydrofluor (National Diagnostics, Somerville, NJ), to give an efficiency that always exceeded 95%, as estimated by both an external standard and the energy distribution of the photons produced. Background radiation, in samples containing no added radiolabel, was determined to be 30 ± 4 cpm. Radioactivity was assayed for a period sufficient to ensure an accuracy within ± 5 cpm and was considered to be undetectable when a sample contained <40 cpm.

Materials. Cysteine, reduced glutathione (GSH), and lambda-phage DNA (sodium salt) were obtained from the Sigma Chemical Co. (St. Louis, MO).

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† Abbreviations: $5NO_2$, 1-methyl-4-phenyl-5-nitroimidazole; 5NO, 1-methyl-4-phenyl-5-nitrosoimidazole; 4NO, 1-methyl-4-nitroso-5-phenylimidazole; GSH, reduced glutathione; 1-[^{14}C]-5NO, 1-[^{14}C]-methyl-4-phenyl-5-nitrosoimidazole; and DMSO, dimethylsulfoxide.

Sodium salts of the polydeoxynucleotides poly[d(A-T)·d(A-T)] ($S_{20,w}$ 8.07) and poly[d(G-C)·d(G-C)] ($S_{20,w}$ 11.8) as well as Sephadex G-50 coarse gel filtration beads were obtained from Pharmacia Chemicals (Piscataway NJ).

1-[^{14}C]-5NO was prepared and characterized by the methods used previously to prepare unlabeled material [1]. In this case, however, the reaction was carried out with 16 mg (80 μmol) of 4(5)-phenyl-5(4)-nitrosoimidazole and 5.6 mg (40 μmol, 50 Ci/mol) of [^{14}C]-methyl iodide (ICN Radiochemicals, Irvine CA) in order to maximize the yield of product in terms of radiolabel derived from [^{14}C]-methyl iodide. The yield of 1-[^{14}C]-5NO was found to be approximately 5 mg (70% yield) as determined by the spectral absorbance obtained on a 1-μl aliquot of the DMSO solution [1]. The radioactivity of 1-[^{14}C]-5NO was assayed by liquid scintillation spectrometry. The calculated specific radioactivity (45 ± 7 Ci/mol) was indistinguishable from that of the [^{14}C]-methyl iodide used in the synthesis (50 Ci/mol) and was unchanged when 1-[^{14}C]-5NO was reisolated by preparative liquid chromatography. 1-[^{14}C]-5NO was stored as a stock solution containing 0.2 μCi/μl DMSO (4 mM).

Reaction conditions. Reaction mixtures for the incubation of DNA with 1-[^{14}C]-5NO were prepared from stock solutions with the following characteristics: DNA and polynucleotide solutions were prepared in water; their concentrations were determined spectrophotometrically using the manufacturer's data. The solution of 1-[^{14}C]-5NO was that described above. Solutions of either cysteine or GSH, which were the final additions to the reaction mixture, were prepared immediately before use at ten times the desired concentration in 0.1 M KPO_4, pH 7.0. Reaction mixtures, in a final concentration of 0.01–0.02 M KPO_4 buffer at pH 7.0–7.2, were prepared in a volume of either 50 or 100 μl in 1-ml plastic conical vials and then incubated aerobically at 37°.

Isolation of DNA. Two methods were used to isolate DNA from the reaction mixtures.

Method 1 utilized gel filtration on Sephadex G-50 to separate DNA from compounds of low molecular weight [9]. Sephadex G-50 coarse gel filtration beads (10 g) were prepared for this procedure by combination with 100 ml of 0.01 M KPO_4 buffer, pH 7.2, that also contained 1 mM EDTA, and the mixture was allowed to equilibrate for 3 hr; the procedure was repeated three times. A 3-ml plastic syringe barrel, its tip loosely plugged with glass wool, was then filled with the prepared Sephadex G-50, and the resulting column was packed by centrifugation at 1900 *g* for 5 min at 0–10°; 100 μl buffer was then applied to the top of the column and the column was centrifuged for an additional 5 min. The sample (approximately 100 μl) was then applied to the top of the column. The column was centrifuged as above and the DNA, which appeared in the void volume, was collected in a 1-ml plastic conical vial. The column was then centrifuged for an additional 5 min, and 40–50 μl of additional eluate was collected in another vial. In this procedure >95% of the added DNA or polynucleotide was recovered in the first fraction but only a negligible amount in the second fraction. This fractionation procedure was repeated five or six times (with an overall recovery of 60–80%) to effect complete elimination of radiolabel in the second fraction and ensure the complete separation of DNA from smaller molecules.

Method 2 was a DNA chromatography procedure which used an "Elutip" DNA purification column (Schleicher & Schuell Inc., Keene, NH) prepared and used according to the manufacturer's directions. The column was prepared by treating it first with 2 ml of a high salt solution (HS) consisting of 1.0 M NaCl, 1 mM EDTA and 20 mM Tris–HCl buffer at pH 7.4 and then with 5 ml of low salt solution (LS), which differed from HS only in having 0.2 M NaCl. The reaction mixture (50–100 μl) was dissolved in 3 ml of LS, and the solution carefully loaded onto the column at a rate of less than 0.5 ml/min. After washing the column with 20 ml LS, DNA was eluted (>90% recovery) by the slow addition of 250 μl HS. The column was then treated with 250 μl of HS to elute a second fraction, which usually contained a negligible amount of DNA. In some cases other radiolabeled material was present in this fraction, so the column fractionation was repeated once to effect the isolation of DNA in the first fraction completely resolved from other radiolabeled material.

Aliquots of DNA isolated from the reaction mixture were quantified spectrophotometrically using the manufacturer's absorbance data and assayed for radioactivity as described above. In all cases, the absorbance maximum corresponded to the manufacturer's specifications, and no other absorption above 210 nm was found. The reproducibility of the measurement of specific activity in the isolated DNA was determined by repeated measurement to be ±1 nCi/mg DNA and that of the radiolabel incorporated was ±0.01%; detection of specific activity below 1 nCi/mg and radiolabel incorporation below 0.01% was not possible.

RESULTS

Table 1 indicates that no incorporation of radiolabel was detected when either DNA (*E. coli* lambda-phage) or double-stranded polynucleotides were incubated with 1-[^{14}C]-5NO for less than 2 hr. Indeed, 5NO is quite stable under these incubation conditions, its half-life at 37° under aerobic conditions in the presence of up to 1 mg/ml DNA being indistinguishable from that (540 ± 20 min) measured in phosphate buffer alone [1]. When incubation was continued for 24 hr, a time sufficient for most of the 1-[^{14}C]-5NO to decompose, some radiolabel was detected in poly[d(G-C)·d(G-C)] but none was found in poly[d(A-T)·d(A-T)].

The incorporation of radiolabel into DNA was enhanced when such thiol compounds as cysteine or GSH were added to the incubation mixture (Table 1). Thus, for example, the presence of 5 mM cysteine resulted in the association with *E. coli* lambda-phage DNA of approximately 0.06% of the 1-[^{14}C]-5NO added, and the presence of 2 mM GSH resulted in the incorporation of 0.02% of the radiolabel. In the presence of 5 mM cysteine, the binding of 1-[^{14}C]-5NO to poly[d(G-C)·d(G-C)] was 0.06%, but was only 0.02% to poly[d(A-5)·d(A-T)]. When the reac-

Table 1. Incorporation of radiolabel from 1-[^{14}C]-5NO into DNA and polynucleotides*

DNA	Thiol†	Incubation time (hr)	DNA specific radioactivity (nCi/mg)	Label incorporated (%)
None	C	2	ND‡	ND‡
E. coli λ phage	—	2	ND	ND
E. coli λ phage	G	2	2.9	0.02
E. coli λ phage	C	2	10.5	0.06
Poly[d(A-T)·d(A-T)]§	—	0.5	ND	ND
Poly[d(A-T)·(A-T)]§‖	—	24	ND	ND
Poly[d(A-T)·d(A-T)]§	C	0.5	2.0	0.02
Poly[d(G-C)·(G-C)]	—	0.5	ND	ND
Poly[d(G-C)·d(G-C)]‖	—	24	4.5	0.03
Poly[d(G-C)·d(G-C)]§	C	0.5	6.4	0.06
Poly[d(G-C)·d(G-C)]	C	0.5	7.4 (6.7)¶	0.06 (0.05)¶

* Reaction mixtures in 0.01–0.02 M KPO_4 buffer at pH 7.0–7.2 contained 1.3 ± 0.1 μCi (25 nmol) of 1-[^{14}C]-5NO, a thiol compound as indicated and either DNA or a polydeoxynucleotide, which were added at the following concentrations: *E. coli* λ-phage DNA, 0.75 μg/μl; poly[d(A-T)·d(A-T)], 1.10 μg/μl; poly[d(G-C)·d(G-C)], 1.06 μg/μl. Mixtures were incubated aerobically in a 1-ml plastic conical vial at 37° for the time indicated. Unless indicated otherwise, the volume of the reaction mixture was 100 μl, and DNA was isolated by gel filtration (method 1, Methods).

† Thiol was either 2.0 mM GSH (G) or 5.0 mM cysteine (C), or was not added (—).

‡ ND indicates none detected (≤1 nCi [^{14}C]/mg DNA or ≤0.01% label incorporated).

§ DNA was isolated by DNA chromatography (method 2, Methods).

‖ The reaction mixture contained 0.7 ± 0.1 μCi 1-[^{14}C]-5NO in a 50-μl volume.

¶ DNA sample values are those obtained when first isolated by gel filtration. Subsequently, these DNA samples were re-isolated by DNA chromatography, and the results of this assay are indicated in parentheses.

tion mixture contained 1.3 μCi 1-[^{14}C]-5NO and 5 mM cysteine in buffer, but lacked DNA or polynucleotides (Table 1, first line), no radiolabeled material was found in the DNA fraction.

The last two lines of data in Table 1 indicate that results were indistinguishable whether DNA was isolated by gel filtration or by chromatography, and that there was no significant change in specific radioactivity when poly[d(G-C)·d(G-C)] originally isolated by gel filtration was subsequently reisolated by DNA chromatography. Therefore, both procedures for isolating DNA detect the same radiolabeled material derived from 1-[^{14}C]-5NO; that material appears to be stably associated and perhaps even covalently incorporated into polydeoxynucleotides and DNA.

As observed previously [1], thiol compounds accelerate the decomposition of 5NO in neutral aqueous solution. Either cysteine (5 mM) or ethanethiol (saturated) caused 5NO (1 mM) to decompose almost immediately, as judged both by the loss of UV absorption bands (315, 365 nm) and by the results of analytical reverse-phase liquid chromatography (LC). A water-5NO adduct (*m/e* 206, MH^+) was formed in this reaction, as judged by mass spectral analysis of either the crude reaction mixture or of the main product (formed within an hour and isolated by reverse-phase preparative LC). Similarly, when 5NO was added to a saturated solution of aniline in water, the rate of decomposition of 5NO was increased by the addition of either cysteine or ethanethiol. In the presence of thiols, the hydrolysis products were diminished and a prominent new product appeared. Mass spectroscopy indicated that this product (*m/e* 281, MH^+) was an adduct formed by the direct addition of aniline to 5NO. Under no condition was there evidence for products formed by the addition of the thiol compound itself, or evidence for the formation of either the known compound, 5-aminoimidazole [1, 10], or the 5-hydroxylaminoimidazole, which has not been isolated [11].

DISCUSSION

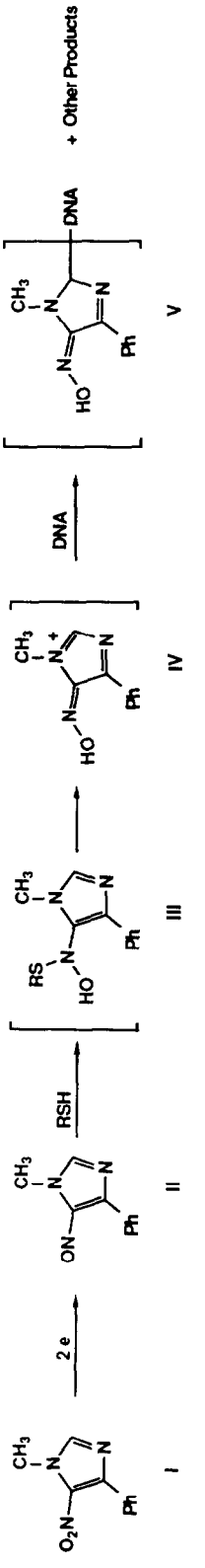
The biological activity of a 5-nitroimidazole apparently results from its conversion to a highly potent, labile species which acts in some way to damage DNA [1–5]. However, neither the nature of the interaction with DNA nor the structure of the reactive species is known. The nitrosoimidazole 5NO has the potency and the lability expected for such an active species [1, 2]; yet the rate of 5NO decomposition in neutral aqueous solution was not increased by the addition of DNA. It must be recognized, however, that biologically significant changes may result from the action of a relatively small number of reactive molecules. Thus, a culture of *E. coli* SR58 at a concentration of 10^8 bacteria/ml sustains a 90% decrease in viability when exposed to 10^{-7} M 5NO under conditions that cause 5NO to decompose rapidly [1]. If one molecule of 5NO is sufficient to kill a bacterium in this assay, then only 1 in 10^6 molecules of the added 5NO would be sufficient for the observed bactericidal effect; even if a thousand 5NO interactions were required to kill a cell, the observed effect would require only 1 in 10^3 molecules of 5NO. Clearly, such small changes would not be detected by measuring the stability of 5NO.

Other methods of detecting interactions with DNA are more sensitive. Thus, it is relatively easy to detect a small number of strand breaks in supercoiled DNA, in which only one strand break results in the formation of open circular DNA, and only one double-strand break results in the formation of linear DNA [12, 13]. Neither of these types of DNA damage was detected, however, when supercoiled ϕX174 DNA was treated with 1 mM 5NO at 37° for times up to 3 hr in the absence of added thiol compounds (Ehlhardt, unpublished data). This result, which in this case excludes the possibility of strand breaks caused by as few as 1 in 10^6 molecules of 5NO, may be compared with the biological results reported when 5-nitroimidazoles were reduced in the presence of ϕX174 DNA [14].

Another sensitive test involves determining the extent to which radiolabel from 5NO becomes associated with DNA when the two are incubated *in vitro*. Under the conditions we used, it is possible to detect the DNA-associated radiolabel from as few as 1 in 10^4 (0.01%) molecules of 5NO. No detectable radiolabel from 1-[^{14}C]-5NO was associated with DNA, however, when it was incubated for less than 2 hr with *E. coli* lambda-phage DNA, poly[d(A-T)·d(A-T)], or poly[d(G-C)·(G-C)]. Because 5NO is relatively stable in this medium ($T_{1/2}$, 540 min), we extended the incubation time to 24 hr, but still found only 0.03% of the 1-[^{14}C]-5NO incorporated into poly[d(G-C)·d(G-C)] and no radiolabel incorporated into poly[d(A-T)·d(A-T)]. Even the larger incorporation into poly[d(G-C)·d(G-C)] (about 3 out of 10^4 molecules of 5NO after 1440 min) is not sufficient, however, to explain the bactericidal effect of 5NO, which, as outlined above, requires the interaction from a minimum of 1 of 10^6 molecules of 5NO within 2 min [1]. These comparisons tend to exclude any direct interaction between 5NO and DNA as the cause of the bactericidal effect of 5NO.

The nitrosoimidazole might still be an intermediate in the bioactivation of nitroimidazoles, if an additional reaction were required to transform it to the species which reacts with DNA. We therefore considered Fig. 1 as a possible outline of the biological activation of $5NO_2$ and thus a general model for the activation of therapeutic 5-nitroimidazoles. The first step of the scheme is the reduction of $5NO_2$ (I) to yield 5NO (II) [3, 4], a compound previously shown to have a number of biological properties expected for the active form of the nitroimidazole [1, 2].

The second step of the scheme is the rapid reversible reaction of II with a thiol compound to form a thiol-nitrosoimidazole adduct (III). This intermediate is comparable to that postulated to explain the reaction between thiol compounds and nitroso-substituted aryl compounds [6–8], which results in the formation of sulfinanilides and the hydroxylamine and/or amine reduction products. A considerable difference would be expected, however, between the reactions of the heteroaromatic thiol-nitrosoimidazole intermediate (III) and those of an aromatic thiol-nitrosoarene adduct. It is known that nitro group reduction of a nitroimidazole renders the imidazole ring more susceptible to hydrolysis [1, 3–5, 11, 12, 15–19]. In an aqueous solu-



Thiol-mediated nitrosoimidazole binding to DNA 7

Fig. 1. A proposal for the activation of $5NO_2$. Proposed intermediates are in brackets. The symbol Ph is used to represent a phenyl group. Further explanation is in the text.

tion containing a thiol, our preliminary evidence suggests that 5NO forms an adduct with water. And when aniline, another good nucleophile, is also present, the analogous aniline-5NO is formed. Rapid formation of a thiol adduct (III) is in accord with the enhanced rate of decomposition of 5NO in the presence of either 5 mM cysteine ($T_{1/2}$ <1 min) or bacteria and mammalian cells and is also consistent with the short-lived, but potent biological activity of the nitrosoimidazoles [1, 2].

It is also possible that the thiol acts to reduce the nitrosoimidazole to the hydroxylaminoimidazole, which then reacts with DNA. We consider this possibility an unlikely explanation for the thiol effect, however, because the thiols react extensively (and rapidly) to form a 5NO-water adduct. Although it is impossible to exclude the formation of a small amount of hydroxylaminoimidazole in this reaction, which then reacts preferentially with DNA, such a reaction conflicts with what is known about the reactivity of hydroxylaminoimidazoles [11, 16, 17, 20].

Our finding that a thiol compound catalyzed the incorporation of radiolabel from 1-[^{14}C]-5NO into both *E. coli* lambda-phage DNA and synthetic double-stranded polynucleotides is in accord with the model reaction described in Fig. 1. Although the product of the reaction with the nucleotides is not defined, it seems likely that a nucleophilic DNA base would attack III preferentially at C_2 to give, at least initially, a structure such as V, either directly or through another electrophilic intermediate (IV) [6, 15]. The finding that poly[d(G-C)·d(G-C)] was more reactive than poly[d(A-T)·d(A-T)] is therefore of interest because it suggests that III or IV may react preferentially with DNA at a guanine base, as occurs when other electrophiles react with DNA bases [20–22]. (The suggested reaction scheme might be modified in the case of such therapeutic 5-nitroimidazoles as metronidazole, whose unsubstituted C_4 may be the site of nucleophilic attack rather than C_2.)

Our data, as interpreted in Fig. 1, are also quantitatively consistent with the available evidence on the biological activity of 5NO. Thus, the concentrations of either cysteine (5 mM) or GSH (2 mM) used in these experiments correspond to thiol concentrations found intracellularly [23, 24]. In addition, the incorporation of radiolabel from 5NO into DNA (6 of 10^4 molecules of 5NO) is more than the minimum fraction of 5NO (1 of 10^6 molecules) that must interact with a bacterium to account for the bactericidal activity of 5NO. (Indeed, the interaction between the reaction form of 5NO and DNA may occur with increased efficiency intracellularly where such intermediates as III or IV may form in close proximity to DNA.)

The relative biological potencies of the 2-, 4-, and 5-nitroimidazoles have been explained by the relative ease with which the initial step in nitro group reduction is accomplished [25–27]. However, a difference in the rate of conversion of I to II (Fig. 1) does not explain why 5NO has 10-fold greater biological potency than 1-methyl-4-nitroso-5-phenylimidazole (4NO) [1, 2]. Such a difference would be expected, however, because intermediate IV should

VI VII

Fig. 2. Possible electrophilic intermediates derived from the thiol catalyzed reaction of a 4-nitroimidazole (VI) and a 2-nitroimidazole (VII).

be more susceptible to nucleophilic attack than its counterpart, VI (Fig. 2), derived from 4NO. Likewise, if 2-nitroimidazoles are activated according to Fig. 1, formation of the comparable intermediate, VII (Fig. 2), would explain why 1-methyl-2-nitrosoimidazole is so much more cytotoxic and mutagenic than 1-methyl-2-nitroimidazole, 1-methyl-2-hydroxylaminoimidazole, and 1-methyl-2-aminoimidazole [28].

Our results apparently do not agree with the observed diminished bactericidal effect of a 5-nitroimidazole when a thiol compound is present [29] and the observation that thiol compounds diminish the toxicity of nitroimidazoles [30] (an effect which is much more pronounced for 2-nitroimidazoles than for 5-nitroimidazoles [31]). Thiol compounds, however, may participate in several reactions related to nitroimidazole activation [30], and their net effect is therefore difficult to predict. Thus, the high concentration of thiol compounds in the vicinity of DNA may be sufficient to support the proposed mechanism for damaging DNA, whereas a high concentration of thiol compounds elsewhere either inside or outside the cell [1] may act to decrease nitrosoimidazole toxicity.

We have observed that the amount of radiolabel from 1-[^{14}C]-methyl-4-phenyl-5-nitrosoimidazole incorporated into DNA in the presence of a thiol compound is large enough to explain the bactericidal effect of 5NO. The chemical properties and biological activities of the 5-nitroimidazoles suggest that this observation is the result of a covalent bond forming between a nucleophilic imidazole intermediate and a DNA base.

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