ABSTRACT

Misonidazole, after reduction to the hydroxylamine derivative, was found to react with guanosine in aqueous solution at pH 7. The guanosine product was isolated and was assigned a structure having a new 5-membered ring with a —CH2—CHOH—linkage between the N-1 and N-2 positions of guanine. Removal of the sugar residue from the guanosine product by acid hydrolysis resulted in the corresponding guanine derivative, which was also made by reacting guanine with reduced misonidazole. In aqueous solution at pH 11, the guanine product was quantitatively converted to guanine within 20 min. A number of N-1-substituted 2-nitroimidazoles and 2-nitroimidazoles reacted with guanosine in an analogous manner, giving rise to the same product as misonidazole, indicating that the C-4-C-5 fragment from the imidazoles is involved in the modification. Neither misonidazole nor its amine or hydrazone derivatives reacted with guanosine. Reduced misonidazole reacted with N-2-methyl guanosine, whereas with N-1-methyl guanosine a reaction was not detected. The identity of Structure I was confirmed by comparison with an authentic sample of Structure I that was prepared by reacting guanosine with glyoxal. Reactions such as the modification of guanine provide a possible molecular mechanism for the cytotoxic and neurotoxic properties of mISONIDAZOLE.

INTRODUCTION

Drugs with the ability to sensitize hypoxic mammalian cells to radiation are of considerable current interest (1, 7, 11, 30). One class of compounds that has received much attention in this respect includes the 2-nitroimidazoles, which have been shown to be effective hypoxic cell radiation sensitizers in numerous in vivo and in vitro studies (2, 6, 12, 13, 19, 22, 31). Misonidazole, after reduction to the hydroxylamine derivative, is currently undergoing clinical trials in the radiation therapy of certain types of human tumors (3, 8–10, 15). Another property of these compounds that is of potential clinical significance is that they exhibit a preferential toxicity towards hypoxic cells (4, 5, 12, 18, 23–25). However, neurotoxicity is a severe limitation in the clinical use of these drugs (9, 10, 30). The mechanism(s) of both of these phenomena is not known. The observation that hypoxic cell toxicity requires prolonged exposure to relatively high concentrations of the drug (5) coupled with the detection of metabolites, in which the nitro group has undergone reduction, suggest that the reduction of the NO2 group may be crucial for the development of toxicity (25–29). One possible mechanism for the cytotoxicity is the modification of cellular macromolecules by metabolites produced on reduction of the nitro group (26). The detection of radioactivity associated with the cellular macromolecular fractions, after exposure to [14C]misonidazole, both under in vivo and in vitro conditions (28) supports such a possibility. To further investigate this possible mechanism, we have studied the reduction products of 2-nitroimidazoles with special emphasis on their ability to modify nucleic acid constituents. In the present study, the modification of guanine derivatives by reduced 2-nitroimidazoles is described.

MATERIALS AND METHODS

Misonidazole and demethylmisonidazole were obtained from Dr. Cary Smithen, Roche Products, Ltd., Welwyn Garden City, Hertfordshire, England. Azomycin (2-nitroimidazole) was purchased from Aldrich Chemical Co. (Canada), Ltd., Montreal, Quebec, Canada. SR-2508 was obtained from the Drug Synthesis and Chemistry Branch, NIH, Bethesda, Md. Guanosine, guanine, deoxyguanosine, N2-methylguanosine, and N1-methylguanosine were purchased from Sigma Chemical Co., St. Louis, Mo. [U-14C]Misonidazole was synthesized as described previously (27). [3H]Misonidazole (48 mCi/mmol), prepared by tritium exchange, was obtained from New England Nuclear, Boston, Mass., purified before use by HPLC, and diluted to 0.48 mCi/mmol. [U-14C]Guanosine (New England Nuclear) was diluted to 0.1 mCi/mmol prior to use. Glyoxal (approximately 40% in aqueous solution) was purchased from Fisher Scientific Company, Fairlawn, N. J., and 2,4-DNPH was purchased from Eastman Kodak Co., Rochester, N. Y. The amine and hydrazone derivatives were prepared as described previously (29).

Reduction of the Nitro Compounds. The reduction of the 2-nitro compounds (structures shown in Chart 1) was carried out in aqueous solution (10–1 M) in the presence of zinc dust and ammonium chloride as described previously (29). The course of the reduction was followed by UV absorption spectrophotometry. When the reduction was complete, as indicated by the absence of any UV absorption maximum above 240 nm, the suspension was filtered and the filtrate without additional purification was used for the reaction with guanine derivatives.

Reaction with Guanine Derivatives. The filtrate was added to an equal volume of the nucleoside solution (2 x 10–3 M) in 0.02 M phosphate buffer at pH 7, except where noted. The reaction mixture was kept well stirred at 37° unless otherwise stated. The progress of the reaction was monitored by HPLC and UV spectroscopy, as described below. For guanine, a saturated solution at 37° in 0.02 M phosphate buffer (pH 7) was prepared and used for the reaction with reduced misonidazole.

HPLC. A Waters liquid chromatograph equipped with a Waters Model 440 absorbance detector which was operated at 254 nm was used. A C18 Bondapak column (30 cm x 9 mm) from Waters Associates (Milford, Mass.) with water:methanol:glacial acetic acid (94:5:1) as solvent provided satisfactory separation of the desired products. Peak heights were taken to quantitate the products and to follow the course of the reaction. Whenever UV absorbance spectra or radioactivity measurements were required, 1-ml fractions were collected. For preparative purposes, fractions constituting the desired compound from a number of HPLC runs were pooled, lyophilized, and used as desired.

1 Supported by the National Cancer Institute of Canada.

2 The abbreviations used are: HPLC, high-pressure liquid chromatography; 2,4-DNPH, 2,4-dinitrophenyhydrazine; NMR, nuclear magnetic resonance.
concentrate was filtered and was applied on a Bio-Gel P-2 (200 to 400 mesh) column (40 x 40 cm) previously equilibrated with 0.01 M ammonium carbonate solution. The column was equilibrated with the same solvent, and 30-ml fractions were collected. The fractions containing the guanosine product were pooled and lyophilized. The residue was taken up in 15 ml of water, and 500 µl portions were injected for HPLC analysis. The eluates containing the guanosine product from each HPLC run were collected, pooled, and lyophilized, and the residue was used as desired.

Reaction with 2,4-DNPH. The reagent was prepared by dissolving 0.25 g of 2,4-DNPH in a mixture of 42 ml of concentrated hydrochloric acid and 50 ml of water by warming on a water bath and then diluting the cold solution to 250 ml with the same solvent. One volume of the solution to be tested was added to 1 volume of the reagent. The red-orange precipitate which formed immediately was filtered and was recrystallized from ethyl acetate.

Spectroscopic Studies. UV absorbance spectra were recorded using a Cary 219 double-beam spectrophotometer. Proton NMR analysis was performed by Dr. Arthur Gray, University of Toronto, on the 360-MHz FT system. The mass spectra were obtained by Dr. Schaffer, Morgan Schaffer Corporation, Montreal, Quebec, Canada, at an ionizing voltage of 70 eV and probe temperature of approximately 170°.

RESULTS

The reaction between guanosine and reduced misonidazole was the first reaction to be studied. Results of preliminary studies indicated the formation of only one guanosine product, and that the HPLC conditions described above were adequate for its separation. A typical separation is shown in Chart 2. The distribution of radioactivity in the various fractions when [H]-misonidazole was used for the reaction is also shown in Chart 2. In contrast, with reduced [14C]misonidazole, radioactivity was not detected in the fractions containing the guanosine product. The reaction proceeded faster at 37° than at 21°, and the optimum yield of product was usually obtained at or near neutral pH.

Guanine reacted with reduced misonidazole in an analogous manner. When the reaction mixture was subjected to HPLC analysis under the conditions of Chart 2, the guanine product and guanine appeared in Fractions 13 and 15, respectively. Acid hydrolysis (0.02 N HCl at 100° for 30 min) of the guanosine product yielded a guanine product having the same UV absorption spectra and chromatographic properties as the product of guanine and reduced misonidazole. The guanosine product was found to be stable for weeks when kept in solutions of pH 6 or lower at 0–5°. In solution at pH 9, the product was quantitatively converted to guanosine within 24 hr at room temperature.

The UV absorbance spectra of guanosine and guanine products at different pH values are shown in Chart 3. On the basis of radioactivity ([U-14C]guanosine), the molar extinction coefficients of the guanosine product have been estimated to be 12 x 10³ at 248 nm and 6 x 10³ at 275 nm in aqueous solutions at pH 2.

A comparison of the spectra with the corresponding spectra of guanosine and guanine indicate that the ratio of absorbance at 275 nm to that at 250 nm has undergone a reduction in the spectra of the products. Such a pattern has been observed in N-2-substituted guanine derivatives (20). Thus, the UV absorption spectra of the products suggest that they are N-2-substituted derivatives. The proton NMR spectra of guanosine and the guanosine product are presented in Chart 4. The only difference between the 2 spectra is the presence of 2 singlets, one at δ = 4.84 (1H) and the other at δ = 5.44 (1H) in the spectrum of the guanosine product. On the basis of UV and NMR spectra, Structure Ia in Chart 5 may be assigned to the guanosine product. The 2 singlets may be assigned to the protons designated as a and b in la (Chart 5). One explanation for the lack of splitting is that the dihedral angle between the 2 vicinal protons is such as to give a minimal coupling constant.

The mass spectrum of the guanosine product did not show a peak at m/e 341, corresponding to the molecular ion. A peak at m/e 341, corresponding to the molecular ion.
at m/e 324 was the highest mass observed. Elimination of H₂O (m/e 18) from the (M + 1) ion could give rise to the m/e 324. Other prominent peaks were at m/e 248, 221, 209, 201, 185, 164, and 151. The mass spectrum of the guanine product also did not show a peak corresponding to the molecular ion at m/e 209. The highest mass was at m/e 191, which could arise from the elimination of H₂O (m/e 18) from the parent molecule. Another prominent peak was at m/e 163, which is probably derived by the elimination of CO (m/e 28) from m/e 191. Thus, the mass spectral data were consistent with the proposed structures of the guanosine and guanine products.

Additional evidence for the structure of the product was provided by the reactions of structural analogues of guanosine and misonidazole. The reaction mixture in each case was analyzed by HPLC and UV absorption spectroscopy. With 1-methyl guanosine, there was no evidence for a reaction with reduced misonidazole. On the other hand, with N²-methyl guanosine, a product having the same UV spectral characteristics as did the guanosine product was detected. These results suggest that substitution in the N-1 position of the guanine moiety prevents the reaction with reduced misonidazole while N-2 substitution has no effect. All four 2-nitroimidazoles tested (Chart 1) reacted with guanosine, giving rise to the same product. From these results, it may be concluded that the C-4-C-5 fragment from the reduced imidazole is responsible for the modification of guanosine. Neither the amine nor the hydrazo derivatives of misonidazole reacted with guanosine.

The proposed structure for the guanosine adduct is the same as that suggested by Shapiro and Hackmann (21) for the product of the reaction between glyoxal and guanosine. To confirm the identity, the glyoxal-guanosine adduct was prepared following the procedure of Shapiro and Hackmann. Chart 6 shows the HPLC separation of the guanosine products prepared by the 2 procedures. The UV absorption spectra of glyoxal-guanosine adduct in acidic (pH 2) and neutral (pH 7) aqueous solutions were identical to those shown in Chart 3A. The NMR spectrum of the guanosine product (Chart 4) is similar to that reported earlier for the glyoxal-guanosine adduct (21) showing only 2 protons as singlets in addition to those of guanosine. The similarities provide confirmatory evidence for the proposed structure.

When a solution of reduced misonidazole was treated with 2,4-DNPH reagent, a red-orange precipitate was formed in
about 3% yield. Its melting point (328°) was identical to that of the 2,4-DNPH derivative of glyoxal. The identification was confirmed by mass spectral analysis, which had an intense peak at m/e 418, corresponding to the molecular ion of 2,4-DNPH derivative glyoxal.

In an attempt to determine whether free glyoxal in the reduction mixture could explain the modification of guanosine, kinetics of formation of the guanosine product from glyoxal and reduced misonidazole was studied. As can be seen from Chart 7, the reaction with glyoxal was complete within 1 hr. With reduced misonidazole, the reaction did not go to completion even after 3 hr. The results may be best explained by postulating an intermediate which releases glyoxal slowly or which reacts directly with guanosine yielding the glyoxal-guanosine adduct.

\[ \text{Chart 6. HPLC separation of glyoxal-guanosine adduct (– – – –) and guanosine product prepared from reduced misonidazole and guanosine (—–). The reactions were carried out in 0.05 M phosphate buffer (pH 7) at 60° for 45 min. Concentrations of the reactants were: glyoxal, 0.55 \times 10^{-3} \text{ M; guanosine, 0.5} \times 10^{-3} \text{ M; misonidazole, 1.25} \times 10^{-3} \text{ M; and misonidazole, 1.25} \times 10^{-3} \text{ M. One hundred-} \mu \text{ l samples were injected into the liquid chromatograph.} \]

\[ \text{Chart 7. Kinetics of formation of glyoxal-guanosine adduct (•) and guanosine product from guanosine and reduced misonidazole (x). The reactions were carried out in 0.05 M phosphate buffer (pH 7) at 60°. Concentrations of the reactants were: glyoxal, 0.5 \times 10^{-3} \text{ M; guanosine (2 \times 10^{-2} M); and reduced misonidazole, 1.25} \times 10^{-3} \text{ M. Fifty-} \mu \text{ l samples were injected at times shown. Absorbance at 254 nm indicates the peak height.} \]

DISCUSSION

Our previous studies (29) on the reduction of misonidazole have shown that reduction of misonidazole with zinc dust in aqueous solution in the presence of ammonium chloride results in the formation of a mixture of the hydroxylamine, the amine, and the hydrazo derivatives. Since neither the amine nor the hydrazo derivative reacted with guanosine, it is to be concluded that the hydroxylamine derivative is responsible for the modification of guanosine. The reactions of guanosine with other hydroxylamines, such as N-2-fluorenylhydroxylamine have been reported previously (16, 17). In these reactions, the hydroxylamine group is directly involved in the addition, and modification at the C-8 position of guanine has been shown to be the major alteration. In the present case, the proposed structure of guanine product suggests that the hydroxylamine group is not directly involved in the addition.

A reaction sequence shown in Chart 8 may be postulated for the formation of the guanosine product from guanosine and reduced 2-nitroimidazoles. The presence of the amine (Chart 8, Structure II), a rearrangement product of the hydroxylamine in the reduction mixture, has been confirmed by the characterization of its dansyl derivative. The low concentration of free glyoxal (3%) and the results of the kinetic studies on the formation of guanosine product may be explained by postulating an intermediate such as Structure IV (Chart 8). Experiments are in progress to characterize the intermediate.

The instability of the guanine product in basic solutions, as demonstrated in the present study, suggests that hydrolytic procedures requiring solutions of pH 8 or higher (such as using alkaline phosphates) are unsuitable for the detection of the guanine product in nucleic acids. Acid hydrolysis appears to be a desirable procedure.

One of the undesirable effects of misonidazole in patients is the development of peripheral neuropathy (9, 10, 30). Since factors such as higher concentrations and longer exposure time increase the incidence of neurotoxicity in the same manner as they enhance cytotoxicity of the drug, it is possible that both

\[ ^3 \text{A. J. Varghese, unpublished data.} \]
have a common mechanism. One possibility is the reductive metabolism which has been shown to be the major metabolic alteration of the drug. The modification of guanine derivatives by reduced 2-nitroimidazoles, as demonstrated in the present study, provides the most direct evidence for a reactive intermediate resulting from the reduction of 2-nitroimidazoles. It should be emphasized that reaction with guanosine is only one of many possible reactions of reduced misonidazole. The formation of other reactive reduction products from 2-nitroimidazoles is also possible. For example, the 14C radioactivity associated with the nucleic acid fractions after exposure to [2-14C]misonidazole cannot be explained by the above mechanism (28). Similarly, it has been reported that electrolytic reduction of misonidazole in the presence of DNA results in the preferential release of thymidine phosphates (14). Nevertheless, the identification of a 2-carbon addition fragment resulting from the interaction of reduced 2-nitroimidazoles with guanine derivatives may have biological or clinical significance, and further studies to examine this possibility are currently under way.

REFERENCES

Modification of Guanine Derivatives by Reduced 2-Nitroimidazoles

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