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# THE RELATIONSHIP BETWEEN MISONIDAZOLE CYTOTOXICITY AND BASE COMPOSITION OF DNA

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**Abstract**—The damage induced by electrolytically reduced misonidazole on DNAs of varying base composition has been measured. Damage assessment using a variety of techniques including viscometry, helix renaturation, hydroxyapatite chromatography and agarose gel electrophoresis indicates that damage is related to A + T content, suggesting that misonidazole cytotoxicity involves a specific target in DNA.

Misonidazole (2-nitro-1-imidazolyl-3-methoxy-2-propanol) is a promising radiosensitizer of hypoxic tumours which is presently undergoing clinical trials [1]. In addition to its radiosensitizing properties this compound has also been shown to exert a cytotoxic effect in both hypoxic and oxic tumour cells [2]. The mechanism of radiosensitization has been studied in some detail [3], but the cytotoxic mechanism remains obscure, although it is known that reduction of the nitro-group is a necessary prerequisite for both these effects.

Recent studies in this laboratory, using a technique involving selective electrolytic reduction of the nitro-group in the presence of DNA, have gone some way towards clarifying the mechanism of cytotoxicity of misonidazole and other nitroimidazoles. The damage induced in DNA by reduced compounds has been measured using a variety of techniques including viscometry, thermal hyperchromicity, melting and renaturation profiles, hydroxyapatite chromatography, agarose gel electrophoresis and sucrose density gradient centrifugation [4–7]. This has established that the major effects of reduced nitroimidazoles are strand breakage of DNA and concomitant destabilization of the helix which results in the formation of single-stranded regions. More recent work has demonstrated that the cytotoxic effect of nitroimidazoles is decreased in the presence of aminothiols [8] and also that the extent of this protection is related to the difference between the reduction potential of the drug and the aminothiol redox couples [9].

We now report that the extent of DNA damage by reduced misonidazole is related to the base composition of the DNA, and in particular the A + T content. This suggests that cytotoxicity involves a specific target rather than random scission of the phosphodiester backbone of DNA.

## MATERIALS AND METHODS

DNAs from calf thymus (type I), *Escherichia coli* (type VIII), *Micrococcus lysodeikticus* (type XI) and *Clostridium perfringens* (type XII) were obtained from Sigma Chemical Co. Ltd., Poole, Dorset, U.K. Poly [d(A–T)] was obtained from the Boehringer Corporation, London, U.K. Misonidazole was a gift from Roche Products Ltd., Welwyn Garden City, Herts, U.K.

Polarography and electrolytic reduction of misonidazole were carried out as previously described [5, 6], as also were viscometric and hydroxyapatite chromatographic analysis of the DNA and agarose gel electrophoresis [6, 7].

Determinations of the mid-point of the helix–coil transition of each DNA (the *Tm* value) and hyperchromicities were made in 15 mM NaCl, 1.5 mM trisodium citrate, pH 7.1 (0.1 SSC) using a Pye Unicam SP 1750 spectrophotometer with a SP 1805 Programme Controller. The cuvette holder was heated electrically from a SP 876 series-2 Temperature Programme Controller at a heating rate of 0.5°. $min^{-1}$. Temperature and absorbance at 260 nm were automatically recorded on a Philips PM 8120 X–Y recorder. Samples were dialysed prior to analysis to remove any absorbing species arising from the drug molecule and to reduce the ionic concentration, which may rise during the electrolytic reduction, to that of 0.1 SSC. No corrections to absorbance readings were made to allow for expansion of the cuvette contents during heating.

The *Tm* values once determined were used to calculate the base composition of each DNA [10, 11] and the values obtained were found to be consistent with those published elsewhere [11]. Since a linear relationship exists between DNA base composition and the maximum hyperchromicity between double- and single-stranded forms [12], this parameter was estimated from the base composition and confirmed experimentally.

Renaturation analysis was carried out as previously described [7] and the percentage renaturation was calculated from the following equation using hyperchromicity data shown in Table 1.

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Table 1.

| Figure reference | Source | % G + C | Hyperchromicity (%) |
|---|---|---|---|
| A | *Clostridium perfringens* | 29 | 44 |
| B | Calf thymus | 40 | 41 |
| C | *Escherischia coli* | 50·5 | 39 |
| D | *Micrococcus lysodeikticus* | 72 | 33.5 |

$$\% \text{ renaturation} = \frac{2(X_{Tm} - X_{50})}{X_{95}\left(1 - \dfrac{1}{1 + \dfrac{hm}{100}}\right)} \times 100,$$

where $X_{Tm}$ is the $A_{260}$ of the DNA at its *Tm*, $X_{95}$ is the $A_{260}$ of the DNA at 95°, $X_{50}$ is the $A_{260}$ of the DNA at 50° after cooling from the *Tm*, and *hm* is the hyperchromicity.

The percentage single-strandedness (% s.s) from the hydroxyapatite chromatography results was calculated using the equation:

$$\% \text{ s.s.} = \frac{\dfrac{A_{260}E_1}{1 + \dfrac{hm}{100}}}{\dfrac{A_{260}E_1}{1 + \dfrac{hm}{100}} + A_{260}E_2} \times 100,$$

where $A_{260}E_1$ is the $A_{260}$ of a 0.12 M sodium phosphate eluate, $A_{260}E_2$ is the $A_{260}$ of a 0.4 M sodium phosphate eluate and *hm* is the hyperchromicity as above.

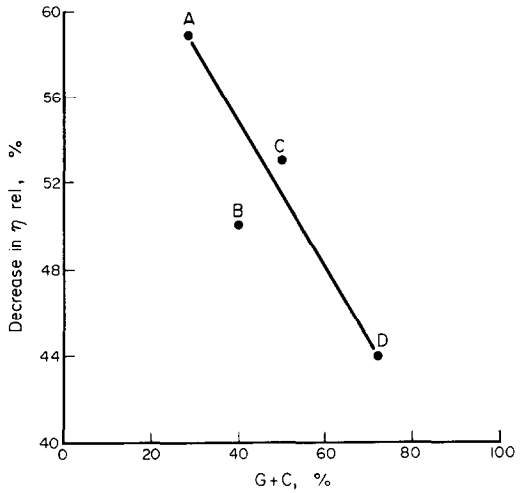


Fig. 1. The effect of reduced misonidazole on the percentage decrease in relative viscosity of DNAs of different base composition. The letters refer to the source and base composition of the DNAs as given in Table 1. Viscosity was measured using an Ubbelohde-type miniature suspended-level viscometer at 30° ± 0.01°. The reduced drug-nucleotide ratio in each case is 1.0.

## RESULTS AND DISCUSSION

The results of this study provide further insight into the mechanism of misonidazole cytotoxicity by showing that the degree of damage incurred by DNA is directly related to its base composition. Thus, when damage is measured as a decrease in relative viscosity ($\eta_{rel}$) of the DNA solution a general trend is observed in which damage to *Cl. perfringens* DNA is more extensive than that to *M. lysodeikticus* DNA (Fig. 1). A decrease in viscosity provides an indication of general, unspecified damage to DNA, however, and if more specific techniques are employed the trend becomes clearer. This is apparent in Fig. 2 which shows the results of renaturation experiments designed to measure the amount of intact helix present in the DNA.

Confirmation of the linear nature of the relationship between damage and DNA base composition comes from the results of the hydroxyapatite chromatographic analysis (Figs. 3 and 4) which measure the increase in single-strand content of the DNA by local unwinding at a site of single-strand breakage.

Results from similar experiments using poly [d(A–T)] indicate that this nucleotide polymer is most

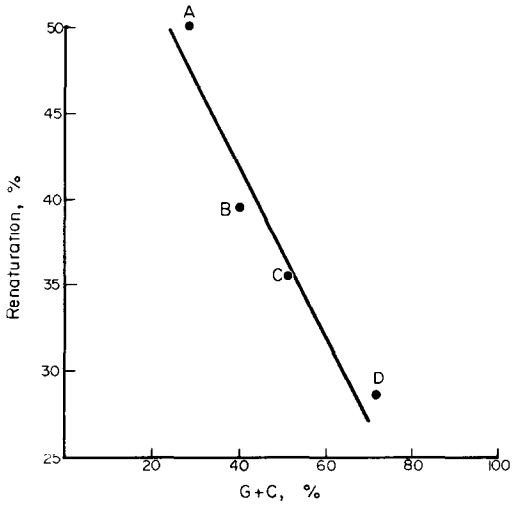


Fig. 2. The effect of reduced misonidazole on the renaturation of DNAs of different base composition. The letters refer to the source of the DNAs as given in Table 1. Renaturation was measured spectrophotometrically as previously described (Rowley *et al.*, 1979).

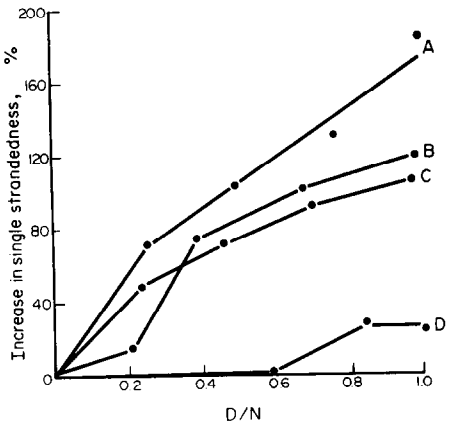


Fig. 3. The effect of reduced misonidazole on the single strand content of DNAs of different base composition as measured by hydroxyapatite chromatography. The letters refer to the DNA source as given in Table 1 and D/N refers to the reduced drug–nucleotide ratio.

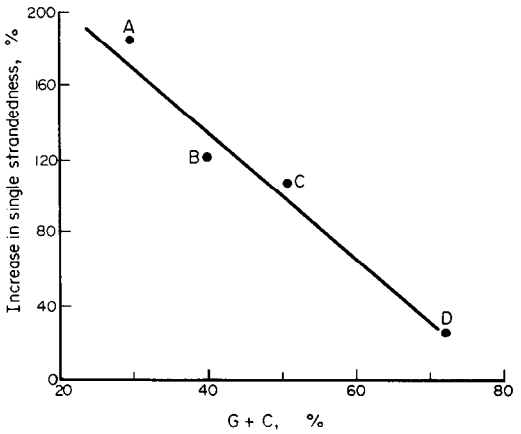


Fig. 4. The relationship between reduced misonidazole-induced damage and DNA base composition. The DNAs may be identified by reference to Table 1.

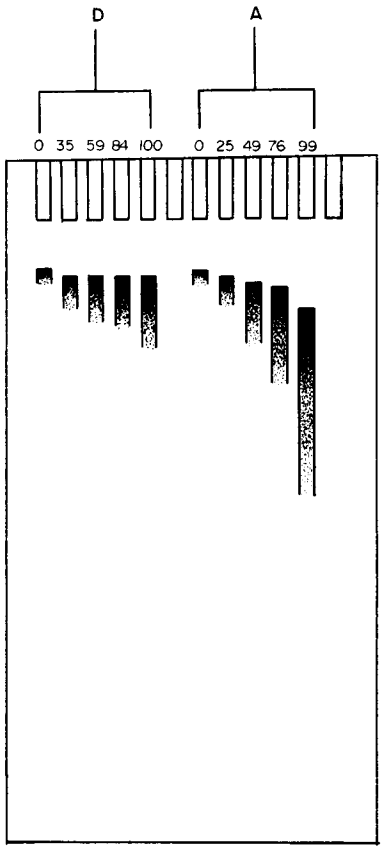


Fig. 5. The effect of reduced misonidazole on the electrophoretic migration of DNAs in acridine-impregnated agarose gels. The gels (1.5%) were electrophoresed at 12.5 mA for 20 hr. The numbers refer to the percentage reduction of misonidazole or (if divided by 100) the reduced drug–nucleotide ratio. The letters refer to the DNA source as given in Table 1. The schematic diagram is drawn to scale.

susceptible to the damaging effects of reduced misonidazole. However, since the buffer conditions used here were different to those used in the study of natural DNAs, direct quantitative comparisons are questionable.

When damage is assessed as a decrease in molecular weight of DNA as measured by the migration distance of the DNA in agarose gels, corroborative evidence of our findings is obtained. An increased migration rate and an increase in band width indicate a lower molecular weight average and an increased polydispersity or molecular weight range of the DNA, respectively [13–17]. In addition, since the gels contain the fluorescent intercalator acridine orange, a decrease in the fluorescence intensity under U.V. light indicates a loss in intact helix related to the ability of the acridine to intercalate the DNA molecule [14–17]. As is shown in Figs. 5 and 6, all these effects are observed in DNA exposed to reduced misonidazole. The decrease in molecular weight is attributable to double-strand breakage which probably occurs as a result of extensive single-strand breakage.

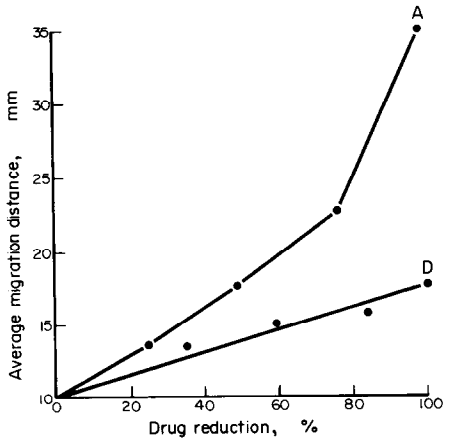


Fig. 6. The effect of reduced misonidazole on the electrophoretic migration of *Clostridium perfringens* (A) and *Micrococcus lysodeikticus* (D) DNA. The numbers refer to the percentage drug reduction or (if divided by 100) the reduced drug–nucleotide ratio.

It is evident from these results that the cytotoxic action of misonidazole on DNA does not involve random strand breakage but rather cleavage at a specific site in the adenine and/or thymidine residues of the DNA. Preliminary studies (to be published elsewhere) indicate that the target is thymidine since thymidine phosphates are specifically cleaved from intact DNA suggesting an action not unlike that of bleomycin [18]. It is clear that such a cytotoxic mechanism would also enhance the radiation-induced damage since radiosensitization has been shown to involve preferential damage to thymidine residues [19].

Since other electron affinic nitroimidazoles have been shown to possess an identical mode of action [4–7] it is likely that this base specificity may be a general feature of radiosensitizing nitroimidazoles. It has not escaped our attention that the relationship between DNA damage and A + T content is a contributory factor in the selective toxicity of these drugs against infections caused by anaerobes. The most important organisms clinically in this respect are the protozoa *Trichomonas vaginalis* and *Entamoeba histolytica* which have A + T contents of 71 per cent and 62–78 per cent, respectively [20–23] and the bacteria *Bacteroides* and *Clostridia* with A + T contents of 59 and 73 per cent, respectively [10, 24]. All these organisms are very susceptible to metronidazole, having minimum inhibitory concentrations of 1 $\mu g.cm^{-1}$ or less. In contrast, *Rhodospirillum* and *Rhodopseudomonas* have A + T contents of 38.4 and 33 per cent, respectively [10, 25] and are approximately 25 times less sensitive to nitroimidazoles [26, 27].

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## REFERENCES

1. S. Dische and G. E. Adams, in *Current Chemotherapy* (Eds. W. Siegenthaler and R. Luthy), pp. 1191–1192. American Society for Microbiology, Washington (1978).
2. J. M. Brown, N. Y. Yu, M. J. Cory, R. B. Bicknell and D. L. Taylor, *Br. J. Cancer* **37** (Suppl. 3), 206 (1978).
3. P. Wardman, *Curr. Topics Radiat. Res. Qt.* **11**, 347 (1977).
4. D. I. Edwards, R. C. Knight and I. Kantor, in *Current Chemotherapy* (Eds. W. Siegenthaler and R. Luthy), pp. 714–716. American Society for Microbiology, Washington (1978).
5. R. C. Knight, I. M. Skolimowski and D. I. Edwards, *Biochem. Pharmac.* **27**, 2089 (1978).
6. R. C. Knight, D. A. Rowley, I. M. Skolimowski and D. I. Edwards, *Int. J. Radiat. Biol.* **36**, 367 (1979).
7. D. A. Rowley, R. C. Knight, I. M. Skolimowski and D. I. Edwards, *Biochem. Pharmac.* **28**, 3009 (1979).
8. E. J. Hall, M. Astor, C. Geard and J. Biaglow, *Br. J. Cancer* **35**, 809 (1977).
9. D. I. Edwards, D. A. Rowley, R. J. Knox, I. M. Skolimowski and R. C. Knight, in *Current Chemotherapy and Infectious Disease.* (Eds. J. D. Nelson and C. Grassi), American Society for Microbiology, Washington pp. 561–563 (1980).
10. J. Marmur and P. Doty, *J. molec. Biol.* **5**, 109 (1962).
11. M. Mandel and J. Marmur, in *Methods in Enzymology* (Eds. L. Grossman and K. Moldave), Vol. 12B, pp. 195–206. Academic Press, New York.
12. H. R. Mahler, B. Kline and B. D. Mehrotra, *J. molec. Biol.* **9**, 801 (1964).
13. C. Aaij and P. Borst, *Biochim. biophys. Acta* **269**, 192 (1972).
14. P. Guerry, D. J. LeBlanc and S. Falkow, *J. Bacteriol.* **116**, 1064 (1973).
15. L. W. Mayer, K. K. Holmes and S. Falkow, *Infect. Immun.* **10**, 712 (1974).
16. J. A. Meyers, D. Sanchez, L. P. Elwell and S. Falkow, *J. Bacteriol.* **127**, 1529 (1976).
17. P. H. Johnson and L. I. Grossman, *Biochemistry* **16**, 4217 (1977).
18. W. E. G. Muller and R. K. Zahn, *Prog. Nucleic Acid Res. molec. Biol.* **20**, 21 (1977).
19. K. D. Asmus, D. J. Deeble, A. Garner, K. M. Idriss-Ali and G. Scholes, *Br. J. Cancer* **37**, (Suppl. 3), 46 (1978).
20. M. Mandel and B. M. Honigberg, *J. Protozool.* **11**, 114 (1964).
21. A. H. Gelderman, D. B. Keister, I. L. Bartgis and L. S. Diamond, *J. Parasit.* **57**, 906 (1971).
22. A. H. Gelderman, I. L. Bartgis, D. B. Keister and L. S. Diamond, *J. Parasit.* **57**, 912 (1971).
23. R. E. Reeves, T. S. Lushbaugh and F. E. Montalvo, *J. Parasit.* **57**, 939 (1971).
24. J. L. Johnson, *Int. J. Systemat. Bacteriol.* **28**, 245 (1978).
25. R. Rudner, H. S. Shapiro and E. Chargaff, *Biochim. biophys. Acta* **129**, 85 (1966).
26. D. I. Edwards, M. Dye and H. Carne, *J. gen. Microbiol.* **76**, 135 (1973).
27. D. I. Edwards, G. E. Mathison and D. J. Platt, *Z. Pflanzenphysiol.* **71**, 424 (1974).