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THE EFFECT OF NITROHETEROCYCLIC DRUGS ON DNA: AN *IN VITRO* MODEL OF CYTOTOXICITY

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Abstract— Electrolytic reduction of several nitroheterocyclic drugs was carried out at controlled potentials under anoxic (anaerobic) conditions in the presence of *Escherichia coli* DNA. The DNA was examined during the reduction process by viscometry, determination of T_m values, and thermal renaturation of DNA; its single strand content was determined using hydroxyapatite chromatography and its electrophoretic migration characteristics in acridine-impregnated agarose gels. All the drugs examined decreased the viscosity, thermal renaturation and T_m value, and increased the single strand content and migration distance of DNA. The results indicate that the drugs cause strand breakage of DNA as a primary mechanism of action, resulting in extensive loss of helix formation and a concomitant decrease in molecular weight. The method of drug reduction and the techniques to assess DNA damage may be used as a model to determine the relative cytotoxicities and the potential of such agents as antimicrobial and radiosensitizing drugs.

Nitroheterocyclic drugs of clinical value include the nitrofurans and nitroimidazoles, with the latter attracting current interest because of their wide clinical applications as antimicrobial agents and their potential as specific radiosensitizers of hypoxic cell tumours. Thus, metronidazole (a 5-nitroimidazole) is in clinical use for a wide variety of protozoal diseases [1–4] and infections caused by sporing and non-sporing anaerobic bacteria [5–7], and is in experimental use as a specific radiosensitizer of hypoxic cell tumours [8–10]. Other 5-nitroimidazoles such as tinidazole, ornidazole and dimetridazole are used for protozoal and bacterial infections [10–14] and misonidazole (a 2-nitroimidazole) is at present the most potent radiosensitizer of hypoxic tumours with possible clinical application [15, 16].

The clinical efficiency and selective toxicity of such drugs as antimicrobial agents lies in their ability to exert a cytotoxic effect which is specific to anaerobes alone, since only these organisms have redox mechanisms of sufficiently low potential to reduce the nitro group, thereby producing a reduction product or products (as yet uncharacterized) responsible for their strand breaking effects on DNA [17–21].

At present it is generally accepted that the mechanisms of radiosensitization and cytotoxicity characteristic of nitroheterocyclic drugs are different. Although reduction of the nitro group is a prerequisite for both effects, cytotoxicity occurs via enzyme-linked reduction mechanisms in anaerobes [22, 23] which are relatively slow and temperature-dependent, whereas radiosensitization occurs by radiation-induced free radicals, which is a fast and temperature-independent process.

These differences have prompted us to investigate the properties of several nitroheterocyclic drugs which are inactive as cytotoxic agents in anaerobes but which may have potential as radiosensitizers. Both processes are inhibited by so-called radical scavengers or radioprotecting agents such as cysteamine, and the primary biochemical lesion of both processes is strand-breakage of DNA.

We present a study of a model *in vitro* system in which the effects of a number of nitroheterocyclic drugs on DNA are presented and which may be of value in predicting not only their cytotoxicity but also their potential as radiosensitizing agents.

MATERIALS AND METHODS

Chemicals. Metronidazole (1-β-hydroxyethyl-2-methyl-5-nitroimidazole), 8609RP (1,2-dimethyl-4-nitroimidazole) and M&B4998 (1-β-hydroxyethyl-4-nitropyrazole) were a gift from May & Baker Ltd., Dagenham, Essex, U.K. and 4 (5)-nitroimidazole was obtained from the Aldrich Chemical Co., Ltd., Gillingham, Dorset, U.K. *Escherichia coli* DNA type VIII was obtained from the Sigma Chemical Co., Poole, Dorset, U.K. as was sodium phosphate and *tris*-(hydroxymethyl)-aminomethane. Citric acid, sodium chloride, trisodium citrate, sucrose and sodium hydroxide were obtained from Fisons Scientific, Loughborough, Leicestershire, U.K., and ethylenediamine tetra-acetic acid (EDTA), disodium EDTA and agarose from British Drug House Ltd., Poole, Dorset, U.K. Acridine orange and sodium acetate were obtained from Hopkin & Williams, Chadwell Heath, Essex, U.K. and all other chemicals were purchased from Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire, U.K.

Preparation of DNA solutions. E. coli DNA type VIII (10 mg) was dissolved in 10 cm^3 15 mM NaCl, 2 mM citric acid, 1 mM EDTA (pH 7.4) with slow stirring over 24 hr at 4°. The solution was dialysed for 65 hr at 4° against 15 mM NaCl, 1.5 mM trisodium citrate (0.1 SSC), pH 7.4. This dialysed preparation was used for all experimental work.

Polarography and electrolytic reduction. Polarographic analysis of all compounds was performed as

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Table 1. Electrochemical characteristics of nitroheterocyclic drugs

Drug	$E_{\frac{1}{2}}$ (pH 7.0) (mV)	pH dependence (mV)	Reduction voltage (mV)
Metronidazole	−385	$E_{\frac{1}{2}}$ = 0.07− 0.065 pH	−900
8609RP	−475	$E_{\frac{1}{2}}$ = −0.02—0.065 pH	−1000
M&B4998	−500	$E_{\frac{1}{2}}$ = −0.08—0.060 pH	−1000
4(5)-nitroimidazole	−540	$E_{\frac{1}{2}}$ = −0.05—0.070 pH	−1000

described previously [19, 20]. From the resulting half wave potentials ($E_{\frac{1}{2}}$ values) at pH 7.0 reducing voltages for the electrolytic reduction of each compound were selected which ensured complete reduction irrespective of slight pH increases during the process, but which also avoided DNA–mercury interactions which occur below −1.0 V [24]. Details of the voltages selected are shown in Table 1.

Electrolytic reduction of each compound in the presence of DNA was carried out at 25° under O_2-free N_2 at constant voltage (±5 mV) using an Ag–AgCl anode and Hg pool cathode with an operating current of 30 ± 5 μA. During reduction the pH was maintained at 7.4 ± 0.5. The reduction vessel contained 10 mg *E. coli* DNA and 200 μmoles of drug in 67 cm^3 of 0.1 SSC buffer (pH 7.4) giving an unreduced drug–nucleotide ratio of 1.0. At intervals samples were removed to determine the extent of drug reduction and for DNA analysis.

Spectrophotometry. Drug reduction was followed by the decrease in absorption maximum of the $-NO_2$ chromophore. This occurred at 320 nm (metronidazole), 315 nm (8609RP) and 300 nm (4 (5)-nitroimidazole), but in the case of M&B4998 (λ max 280 nm) the absorption decrease was monitored at 300 nm where no significant interference occurred from the absorption of DNA (λ max 260 nm).

Viscometry and agarose gel electrophoresis. These were performed as described previously [19, 20] as was hydroxyapatite chromatographic analysis of double and single strand components of DNA [20]. Prior to the chromatographic and thermal renaturation analysis of DNA the samples (5 cm^3) were dialysed against 125 cm^3 0.1 SSC buffer (pH 7.4) at 4° overnight. This step reduces the ion concentration (mainly from the anode) which raises the value of the mid-point of the DNA helix-coil transition, (the T_m value) as a result of increased ionic stabilization [25–27]. The dialysis also removes any bound drug molecules which may affect binding of DNA to hydroxyapatite.

Thermal renaturation analysis. This method relies on one basic assumption, namely that the DNA has been totally transformed into its single-stranded state at temperatures where the melting profile shows its upper plateau. Under the experimental conditions employed this point was judged to have been reached at a temperature of 95°.

If the absorbance at 260 nm of the DNA solution at 95° is denoted as X_{95} and the theoretical maximum hyperchromicity (h_m) between solutions of zero and 100% single-strand content for the type of DNA under analysis is known, a theoretical value for the absorbance of an identical solution containing 100% double-stranded DNA (X_t) can be calculated.

$$X_t = \frac{X_{95}}{1 + (h_m/100)} \tag{1}$$

At the melting temperature (T_m) the single-strand content of a DNA solution is exactly half way between its original state and 100% single-strandedness. For the theoretical case this represents 50% single-strandedness and the maximum hyperchromicity between 0% single-strandedness and this point ($\Delta X_{\frac{1}{2}}$) can be calculated.

$$\Delta X_{\frac{1}{2}} = \frac{X_{95} - X_t}{2} \tag{2}$$

This value equally represents the theoretical maximum amount of *hypochromicity* which could be observed on cooling a solution of DNA from its T_m.

For an experimental sample the baseline absorbance before heating will vary according to the condition of the DNA but the final result after total thermal denaturation will be a solution containing 100% single-stranded DNA. The absorbance of this final solution will vary as a function of DNA concentration and the ionic strength of the buffer but in each case X_t can be calculated, and from that $\Delta X_{\frac{1}{2}}$. This value can then be directly compared with the amount of renaturation observed on cooling an identical sample from its T_m to give a measure of the amount of renaturable DNA in the sample.

If the absorbance of the sample at its T_m is denoted X_{Tm} and its absorbance after cooling, which under the conditions employed was found to have reached a stable level at 50°, is denoted X_{50}, then:

per cent of renaturable DNA in sample

$$= \frac{X_{Tm} - X_{50}}{\Delta X_{\frac{1}{2}}} \times 100 \tag{3}$$

Substituting (1) in (2)

$$\Delta X_{\frac{1}{2}} = \tfrac{1}{2}\left(X_{95} - \frac{X_{95}}{1 + (h_m/100)}\right)$$

$$= \frac{X_{95}}{2}\left(1 - \frac{1}{1 + (h_m/100)}\right)$$

For *E. coli* DNA h_m = 39%

$$\therefore \Delta X_{\frac{1}{2}} = X_{95} \times 0.1403$$

Substituting in (3)

$$\text{per cent renaturable DNA} = \frac{X_{Tm} - X_{50}}{0.1403 \times X_{95}} \times 100$$

Experimentally, three parameters, X_{95}, X_{Tm} and X_{50}, must therefore be measured and for the equation to hold

true these measurements must be made on identical solutions.

Thermal denaturation and renaturation profiles were obtained as described previously [20] with half a sample being used to obtain values for the T_m and X_{95} and the other half yielding values of X_{Tm} and X_{50}.

RESULTS AND DISCUSSION

Polarographic analysis of all drugs gave a single polarographic wave. The half-wave potentials ($E_{\frac{1}{2}}$) against the Ag–AgCl electrode are pH-dependent and obey the equations shown in Table 1 between pH 2.0 and 8.5.

The effects of the drugs on the viscosity of the DNA solution are shown in Fig. 1. The decrease in relative viscosity as the reduced-drug nucleotide ratio increases can be attributed to several events including strand separation, strand breakage or 'bending' of the helix [28]. The results for the effects of the drugs on the T_m value of DNA (Fig. 2) and the amount of intact helix in the molecule (Fig. 3) also show a progressive decrease as the reduced-drug concentration is increased, and these effects may also be attributed to strand breakage or strand separation. DNA bending does not alter the helix content of the molecule.

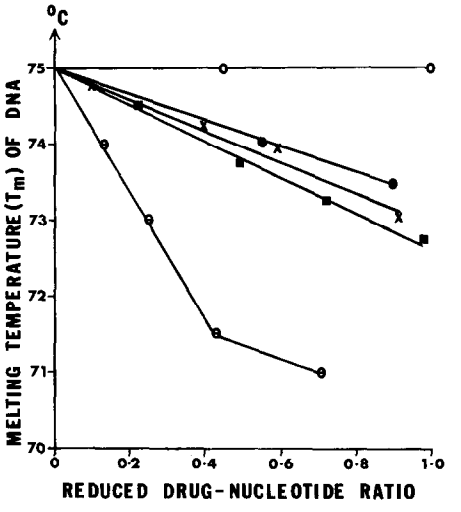

Fig. 2. The effect of reduced nitroheterocyclic drugs on the melting temperature (T_m) of *E. coli* DNA. ○——○, DNA exposed to a reducing current in the absence of any drug; ●——●, in the presence of metronidazole; ×——×, in the presence of 4-nitroimidazole; ■——■, in the presence of 8609RP; ⊖——⊖, in the presence of M&B4998. The estimated position of the DNA control curve is based on the average amount of drug reduction occurring over an equivalent time period.

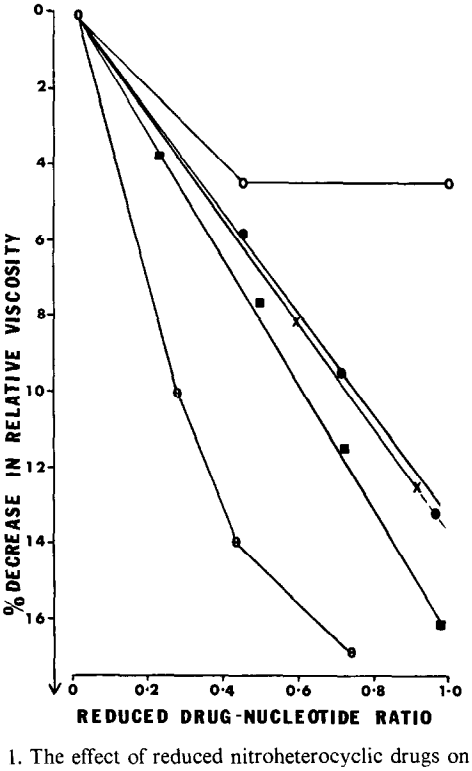

Fig. 1. The effect of reduced nitroheterocyclic drugs on the relative viscosity of *E. coli* DNA. All viscometric measurements were made at 30° ± 0.01°. ○——○, DNA exposed to a reducing current in the absence of any drug; ●——●, in the presence of metronidazole; ×——×, in the presence of 4-nitroimidazole; ■——■, in the presence of 8609RP; ⊖——⊖, in the presence of M&B4998. The estimated position of the DNA control curve is based on the average amount of drug reduction occurring over an equivalent time period.

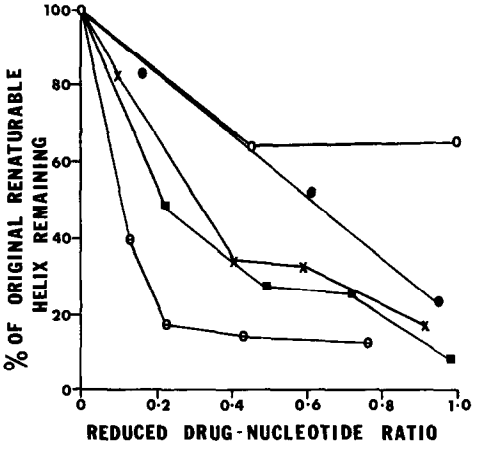

Fig. 3. The effect of reduced nitroheterocyclic drugs on the renaturable double helix content of *E. coli* DNA. Samples were heated to the T_m and cooled to 50°. Helix content was calculated assuming 39% hyperchromicity reflects a 100% double helix content. Results are shown as the percentage drop in helix content relative to the initial (unreduced) sample. ○——○, DNA exposed to a reducing current in the absence of any drug; ●——●, in the presence of metronidazole; ×——×, in the presence of 4-nitroimidazole; ■——■, in the presence of 8609RP; ⊖——⊖, in the presence of M&B4998. The estimated position of the DNA control curve is based on the average amount of drug reduction occurring over an equivalent time period.

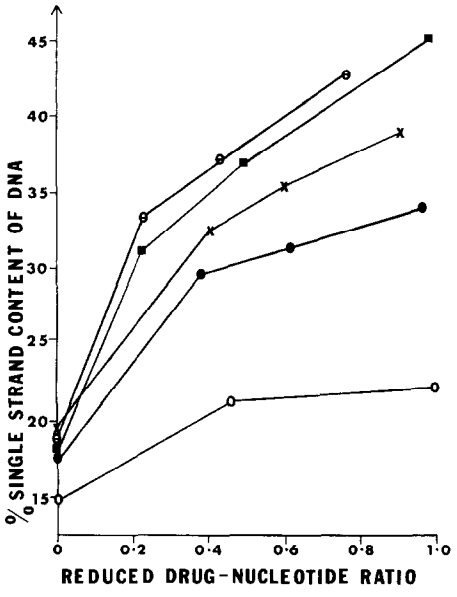

Fig. 4. The effect of reduced nitroheterocyclic drugs on the single strand content of *E. coli* DNA. Single and double stranded DNA in the samples were separated on hydroxyapatite columns at 60° after mild sonication. The quantities of DNA in each fraction were determined spectrophotometrically at 260 nm. O----O, DNA exposed to a reducing current in the absence of any drug; ●——●, in the presence of metronidazole; ×——×, in the presence of 4-nitroimidazole; ■——■, in the presence of 8609RP; ⊖——⊖, in the presence of M&B4998. The estimated position of the DNA control curve is based on the average amount of drug reduction occurring over an equivalent time period.

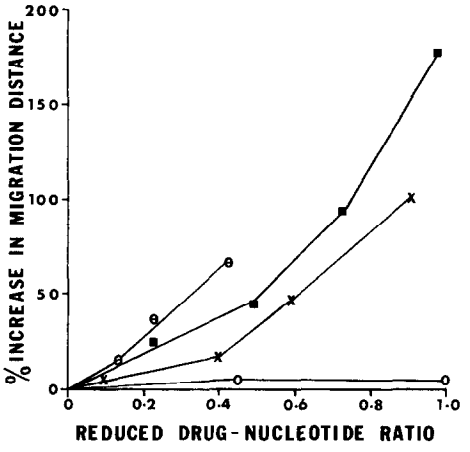

Fig. 5. The effect of reduced nitroheterocyclic drugs on *E. coli* DNA migration in agarose gels. Samples were electrophoresed in the presence of the intercalating dye acridine orange allowing determination of band position by fluorescence under short wave U.V. light. The mid-point of each band was used in calculating the percentage increase in migration distance relative to that of the unreduced sample. O——O, DNA exposed to a reducing current in the absence of any drug; ×——×, in the presence of 4-nitroimidazole; ■——■, in the presence of 8609RP; ⊖——⊖, in the presence of M&B4998. The estimated position of the DNA control curve is based on the average amount of drug reduction occurring over an equivalent time period.

The relative amounts of single stranded and double stranded DNA in any preparation may be quantitatively resolved by hydroxyapatite chromatography, and Fig. 4 indicates that the drugs produce a progressive increase in the single strand content of DNA. Although this effect taken alone may be interpreted as the result of single-strand breakage within the double helix, or strand separation, it can be seen that the effect decreases at high reduced-drug concentration and levels out at a single strand content of 35–45%. If these values are compared with the renaturation data of Fig. 3 which show a final double helix content as low as 10% it is obvious that strand separation alone cannot account for the action of these drugs since a much closer correlation between the two sets of results would be expected. That the primary mechanism of action is strand breakage can be explained in the type of breakage occurring. Thus, single strand breakage within the double helix would render the DNA more susceptible to strand separation under the conditions of mild sonication employed prior to hydroxyapatite chromatography, whereas double strand breakage would have the opposite effect and both types of breakage would reduce the capacity of DNA to renature after thermal denaturation.

Corroboration of such a mechanism, viz. strand breakage as a primary mechanism of action of these drugs, comes from the results of agarose-gel electrophoresis (Fig. 5). As the reduced-drug concentration increases the migration distance of DNA increases. This effect is accompanied by a spreading of the band and a gradual decrease in the fluorescence intensity of intercalated acridine. These observations indicate the formation of DNA of lower molecular weight average, an increase in the molecular weight range of DNA, and a decrease in the overall helix content of the DNA. Samples containing unreduced drugs showed no significant increase in migration distance compared with pure samples of *E. coli* DNA. Other experiments using alkaline sucrose sedimentation ultracentrifugation have also shown that the nitroimidazole drug misonidazole causes strand breaks in DNA [20].

In all these experiments the unreduced drug has no significant effect on the integrity of DNA and the observed effects are only accomplished under anoxic or anaerobic conditions.

In addition to showing that a wide variety of nitroheterocyclic agents strand break DNA as a primary mechanism of action the results also demonstrate their relative efficiency as strand breakers. Thus, the relative toxicity of these agents to DNA is in the increasing order metronidazole, misonidazole [20] 4-nitroimidazole, 8609RP, and M&B4998 — and this order remains unchanged irrespective of the technique used to measure DNA damage. This order also correlates with the known relative cytotoxicities of metronidazole and misonidazole (that of the others has not been established).

If the order of effectiveness in damaging DNA is compared with their ability to accept electrons (i.e. their reducibility or $E_{\frac{1}{2}}$ values; Table 1) it is immediately apparent that no obvious correlation exists. This is interesting since it has been demonstrated that radiosensitization can be correlated with the one-electron

redox potentials of nitroheterocyclic drugs [29]. This is expected if the radiosensitization capacities and cytotoxicities reflect different mechanisms, but not if both processes involve identical mechanisms. Our reduction system is not solely a one electron process, since a variety of compounds have been detected amongst the reduction products of the drugs, including the amine (unpublished data). That DNA damage in our system is a reasonable model of cytotoxicity can be judged from the fact that the process is affected by temperature and occurs by way of a free radical mechanism since it is inhibited by cysteamine (data to be published elsewhere). Thus, the DNA damage detected in our model includes all the known parameters of cytotoxicity in intact cells.

Our data therefore indicate that whereas radiosensitization may be correlated with one-electron potentials the cytotoxicity of the drugs may not, and this lends support to the hypothesis that radiosensitization and cytotoxicity proceed via different mechanisms.

However, our *in vitro* model does not take into account several factors which may affect the magnitude of the cytotoxic effect in the intact cell. Thus, the fact that radiation itself can reduce nitroheterocycles under anoxic conditions [30] and that metronidazole inhibits a known DNA repair enzyme [19] may influence the overall extent of DNA damage observed.

Two of the drugs investigated, the 4-nitroimidazole (8609 RP) and the 4-nitropyrazole (M&B4998), are ineffective as antimicrobial drugs because their redox potentials are too low and consequently the nitro group cannot be reduced either by aerobes or anaerobes [23]; but nevertheless these are powerful strand breakers of DNA when reduced. There remains the possibility that radiation could reduce a sufficient amount of the drug to produce DNA damage synergistically, as has been demonstrated with other strand-breaking drugs [31] and this suggests that such compounds should not be ignored as potential radiosensitizers.

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