Biochemical Pharmacology, Vol. 36, No. 19, pp. 3299–3304, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

DNA DAMAGE INDUCED BY REDUCED NITROIMIDAZOLE DRUGS

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(*Received* 27 *January* 1987; *accepted* 28 *April* 1987)

Abstract—Five nitroimidazole drugs were reduced electrolytically and by γ-radiolysis at fast (300 μmoles or 100% per hr) and slow (3–9 μmoles or 1–3% per hr) reduction rates in the presence of *Escherichia coli* DNA and single stranded or double stranded DNA from the bacteriophage ØX174. The degree of DNA damage depends upon the rate of drug reduction, where slow reduction produces more damage than fast reduction. The efficiency of damage produced is in the order metronidazole > ornidazole > azomycin > misonidazole > benznidazole which reveals a linear correlation between the one-electron reduction potential ($E_{\frac{1}{7}}$) and the negative logarithm of the concentration of reduced drug at which 37% of the original DNA activity remains. Damage is not influenced by the presence of O_2 at least between about 1–100 ppm. We suggest the protonated one-electron nitro radical anion as a possible candidate for the active damaging species and explain the basis of the relative cytotoxicity of these drugs under conditions of hypoxia.

The basis for the cytotoxic action of nitroimidazole drugs such as misonidazole and metronidazole lies in their ability to be reduced via the nitro group to unknown products which cause death of susceptible cells. Oxygen inhibits drug reduction due to a futile cycle reaction with the one-electron radical anion which results in the formation of the parent drug and superoxide [1, 2]. The major target for nitroimidazole action is DNA in both anaerobic microorganisms [3, 4] and hypoxic mammalian cells [5].

To study the cytotoxic mechanism we have developed a model of the drug–target interaction in which the nitro group of the drug is reduced at a constant potential in the presence of DNA and the damage produced quantified. These studies have established that reduced nitroimidazoles cause strand breaks and helix destabilization leading to the formation of single stranded regions [6–8]. The damage depends upon the base composition of the DNA [9] and is associated with the specific release of thymidine phosphates in a reaction which does not involve significant binding of the drug to DNA [10].

Using the electrolytic reduction system and reduction by γ-radiolysis we have investigated the effect of a number of nitroimidazole drugs reduced at fast (100% per hour) and slow (1–3% per hr) reduction rates on DNA from *E. coli* and from the bacteriophage ØX174 and correlated the damaging effect with the one electron reduction potential.

These results together with coulometric analysis of the electron requirements for reduction and the effect of pH are presented and a possible mechanism discussed.

MATERIALS AND METHODS

Sources and properties of the drugs used in the study are given in ref. 11. DNA from *Escherichia coli* was obtained from the Sigma Chemical Co. Ltd., Poole, Dorset, and further prepared as previously described [8]. All other chemicals were analytical grade and used without further purification.

Transfection assay. The methods of preparing single-stranded DNA from the bacteriophage ØX174 has been described by Blok *et al.* [12]. The double stranded replicative form of ØX174 DNA was isolated using the method of Baas *et al.* [13]. The double stranded preparation consisted of greater than 95% of the RF1 type (supercoiled) form as shown by sucrose gradient sedimentation [14]. The biological activity of both single and double stranded ØX174 DNA was measured using an *E. coli* transfection assay as essentially described by Blok *et al.* [12] and Lafleur *et al.* [15]. Briefly, usually 0.1 ml of a 0.1 μg/ml solution of ØX174 DNA was mixed with an equal volume of *E. coli* K12 (AB1157) spheroplasts and after 10 min at 20° 0.8 ml of LBM (Luria broth medium) containing 10% (w/v) sucrose and 0.1% (w/v) glucose, 0.2% (w/v) $MgCl_2$ was added and incubated at 37° for at least 2 hr. Subsequently 4 ml of cold distilled water was added and the released phage determined using *E. coli* C as the indicator organism.

As a measure of the sensitivity of DNA the R_{37} value is used which is obtained from the slope of the survival curve by a least squares analysis of the strictly exponential decay of the survival curves of ØX174 DNA.

Electrochemical methods. Details of the polarographic determination of half wave potentials ($E_{\frac{1}{2}}$) and electrolytic reduction of the drugs at constant

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potential have been described previously [6–9]. Generally, the drugs were reduced at *ca.* 550 mV below the E_4 value under N_2 bubbling in vessels shielded from light. The N_2 gas supply contained less than 10 ppm O_2. The reduction vessel contained 300 μM *E. coli* DNA together with a small amount of single or double-stranded ØX174 DNA (at 3×10^{-7}–6×10^{-6} M) at a drug–(*E. coli*) DNA nucleotide ratio of 1 at pH 7.0 ± 0.5. Drug reduction was monitored as the decrease in the absorption maximum of the nitro group and by the decrease in current density from *ca.* 30 μA to zero. The reduction rate was approx. 1.5% drug/hr unless otherwise stated.

The number of electrons involved in the reduction process was measured coulometrically as described previously [16]. In brief, the current flowing during reduction is integrated with time to produce counts on a digital readout. The number of electrons involved in reduction (N) could be calculated to two decimal places.

Radiolytic reduction. Radiolysis was carried out under N_2O with ^{60}Co γ-rays (Gamma cell 100 or 200, Atomic Energy of Canada Ltd.). The drug reduction rate was 2±1% (40 Gy) per hour unless stated otherwise with a DNA concentration of 3×10^{-6} M single-stranded (or double-stranded) ØX174 DNA in 1.3×10^{-2} M phosphate buffer pH 7.0 ± 0.2 and 2×10^{-1} M isopropanol. The oxygen concentration was reduced to less than 2 ppm, by means of an active copper catalyst (BTS catalyst of the Badische Anilin und Soda Fabrik, Ludwigshafen am Rhein, F.R.G.). In the case of these experiments it is important to know the residual oxygen concentration in the nitrous oxide gas explicitly. However, direct measurements of this concentration in N_2O with a Hersch cell is not possible because of disturbing electrode reactions. The oxygen content in nitrogen gas led through the same pathway as the nitrous oxide was therefore measured and found to be contaminated with about 1 ppm oxygen. The N_2O gas was now considered to be contaminated with the same amount of oxygen, assuming that the copper catalyst reduces the oxygen contamination to the same level in both cases. Further, all the results are corrected for the small contribution to the inactivation of the DNA by the remaining unscavenged water-radicals.

Determination of nitrite. Formation of the nitrite ion during reduction was measured by a modified sulphanilimide/*N*-(1-naphthyl)-ethylenediamine reaction as previously described [16].

Viscometry. The specific viscosity of DNA from *E. coli* was measured as previously described [7].

RESULTS

The action of reduced nitroimidazoles on DNA results in the specific release of thymidine derivatives [10] and misonidazole-induced DNA damage has been correlated with the % A+T content of DNA using a wide range of methods to assess DNA damage [9, 10]. Further, it has been previously shown that 2-nitroimidazoles require fewer electrons for complete reduction in the presence of DNA than in its absence [16].

The electron requirement (N) for misonidazole reduction varies with the DNA base composition and this effect correlates well with the amount of thymidine released [10]. If N varies in the presence of DNA the final stable reduction products cannot be responsible for DNA damage or cytotoxicity, but confirms the concept of the cytotoxic agent as a short-lived reduction intermediate.

Figure 1 shows some examples of survival curves of ØX174 DNA during reduction of misonidazole. Here, the effect is determined by the rate of reduction of the drug: (a relatively slow reduction rate produces more damage than a high rate; see also Fig. 2) and the nature of the DNA (single-stranded DNA is more sensitive to damage than double-stranded DNA). Figure 1 also shows that at a reduction rate of 100% (2 kGy) per hr no lethal damage to DNA occurs as a consequence of reduction products. Also, with the radiation induced reduction, a clear increased inactivation of single-stranded ØX174 DNA is found with decreasing reduction rate (see also [17]) resulting in, for example, an $R_{37} = 8\%$ for misonidazole using a reduction rate of 2% per hour which is in line with the data for electrolytic reduction (see also Fig. 2).

The survival curves are strictly exponential and therefore reflect inactivation by single-hit kinetics which implies a Poisson distribution of lethal damage amongst the population of DNA molecules.

The values of one lethal hit (R_{37}) are calculated for 37% remaining activity and the reciprocal ($1/R_{37}$) values reflect directly the relative amount of damage in DNA and enables a direct comparison of drugs to be made (the higher value the more damage).

Similar experiments as shown above were repeated with metronidazole. The data, obtained at relatively slow reduction rates (1–3%/hr) together with those for misonidazole, are summarised in Fig. 2. This figure shows the variation in $1/R_{37}$ with the reduction rate. It is clear that greater damage is

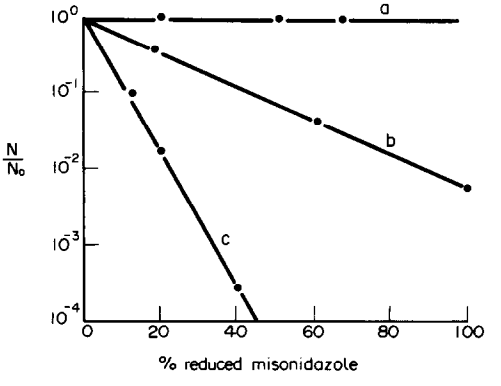


Fig. 1. DNA damage induced by misonidazole at different reduction rates: (a) is the survival curve of single stranded or double stranded (replicative form) ØX174 DNA when misonidazole is radiolytically reduced at 100% per hour at pH 7.0 ± 0.1; (b) is the survival curve of double stranded ØX174 DNA when misonidazole is electrolytically reduced at 1.2% per hour at pH 7.0 ± 0.5; (c) is the survival curve of single stranded ØX174 DNA when misonidazole is electrolytically reduced at 1.2% per hour at pH 7.0 ± 0.5.

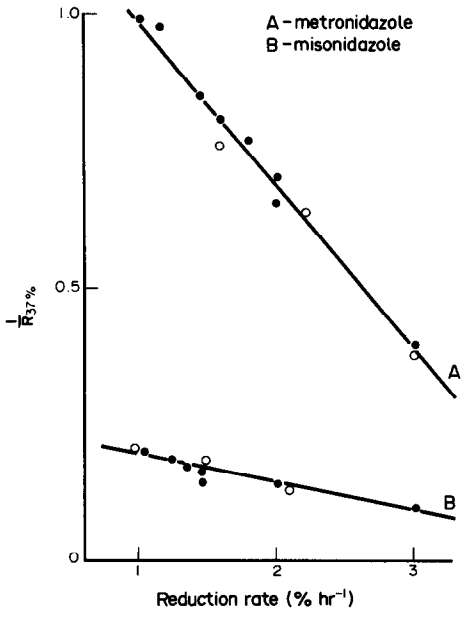


Fig. 2. The effect of different reduction rates of misonidazole and metronidazole when electrolytically reduced on single stranded (●—●) ØX174 damage. In the same figure data for double stranded ØX174 DNA (○—○) are plotted, corrected for the difference in sensitivity between single and double stranded DNA (see Fig. 1). The reciprocal of the R_{37} value directly reflects the degree of DNA damage induced by the reduced drugs. Thus, at a reduction rate of 1% per hr metronidazole causes about five times more damage than misonidazole.

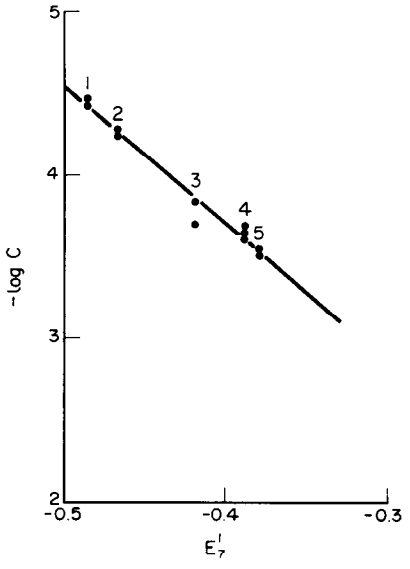


Fig. 3. The relationship between the one-electron reduction potential (E_7^1) of five nitroimidazoles and the negative logarithm of the concentration of reduced drug at which 37% of the original single stranded ØX174 DNA activity remains (—log C). The numbers refer to the drugs as follows: 1—metronidazole, 2—ornidazole, 3—azomycin, 4—misonidazole, and 5—benznidazole.

produced by metronidazole than misonidazole for a given reduction rate but that in both cases little damage to single-stranded ØX174 would be expected if the reduction rate exceeded about 5% per hour. Therefore, apart from the reduction rate, the amount of lethal damage to single-stranded ØX174 DNA also depends on the nature of the drug. Similar results are obtained with double-stranded DNA (as also shown in Fig. 2).

It has been suggested that low levels of O_2 may play an important part in determining the life times of the transient intermediate of the reduction of nitroimidazoles [18]. The results presented indicate that the presence of traces of oxygen (at least up to 100 ppm) seems not to influence the lethal damaging effect of the drug reduced electrolytically (see Fig. 2) because reduction by radiolytic techniques, in the presence of 1 ppm O_2 at a similar slow reduction rate gives about the same inactivation of ØX174 DNA (see above). In fully oxygenated solutions no lethal damage in DNA occurs as a consequence of reduction products, even at low reduction rates (data

Table 1. Effect of pH on nitrite production from misonidazole in the absence (−DNA) and presence (+DNA) of *E. coli* DNA, the variation in the number of electrons (N) required for complete electrolytic reduction of the drug and the effect on *E. coli* DNA damage measured as a decrease in the specific viscosity (hsp)

pH	Molar % NO_2^-		N		% decrease in hsp.
	−DNA	+DNA	−DNA	+DNA	
4.0	1.5	—	—	—	—
4.5	—	4.0	3.96	—	72
5.0	2.0	4.3	3.98	4.00	68
5.5	—	—	4.05	4.06	60
6.0	2.1	4.5	4.18	4.15	59
6.5	—	—	4.21	—	—
7.0	2.0	4.8	4.24	4.20	58
7.5	—	—	4.35	—	—
8.0	2.4	5.4	4.43	4.26	58
8.5	—	—	4.48	4.35	52
9.0	3.0	6.0	4.63	4.47	40
9.5	3.1	—	—	—	31

not shown), probably because of futile cycling [1]. Even a decrease in reduction rate by a factor of 50 (100% to 2% per hr) showed no damage within an accuracy of 5%. Further, the degree of damage to ØX174 DNA during electrolytic reduction is not altered in the presence of catalase, nor when the N_2 gas is passed through alkaline dithionite or an active copper catalyst to decrease the oxygen contamination nor are electron values altered under these conditions.

The damage produced depends also upon the one-electron reduction potential (E_7^1) of the drugs as is shown for five drugs in Fig. 3. Here C is the concentration of reduced drug at which 37% of the activity of the single-stranded ØX174 DNA remains and the linear relationship between $-\log C$ and E_7^1 is given by the equation

$$-\log C = b_0 - b_1 E$$

where

$$b_0 = 0.009 \pm 0.002 \text{ mol dm}^{-3}$$

and

$$b_1 = 9.1 \pm 0.2 \text{ mol dm}^{-3} \text{ V}^{-1}$$

The same sequence of damage is shown during radiolytic reduction of metronidazole > ornidazole > azomycin > misonidazole > benznidazole where the slope, i.e. $b_1 = 9.3 \pm 0.3 \text{ mol dm}^{-1} \text{ V}^{-1}$.

The action of misonidazole is affected by pH. Damage produced by misonidazole, measured as a decrease in the specific viscosity of DNA and strand breakage by filter elution shows an enhancement at acid pH and a concomitant decrease in the electron requirement from about 4.7 at pH 9 to 3.9 at pH 4.5. The small amount of nitrite produced at pH 9 (6%) also decreases to about 4% pH 4.5 (see Table 1 and ref. 19).

DISCUSSION

The relationship between lethal DNA damage as measured by the reciprocal of the R_{37} value and the one-electron reduction potential E_7^1 gives for electrolytic reduction a coefficient of $b_1 = 9.1 \pm 0.2 \text{ mol dm}^{-3} \text{ V}^{-1}$ which indicates that a *decrease* in the reduction potential of 0.1 V results in an order of magnitude *decrease* in the amount of reduced drug required to produce a fixed response. The coefficient for radiolytic reduction of these drugs at a reduction rate of $2 \pm 1\%$ is $9.3 \pm 0.5 \text{ mol dm}^{-3} \text{ V}^{-1}$. These values are similar to that found for strand breakage and thymine release (11 V^{-1}) [20] but has an opposite slope of that found for radiosensitization and other systems [21, 22].

A significant finding is that oxygen is not involved in the damaging process, at least in the concentration range of 1–100 ppm. However, at the surface of the Hg pool probable depletion of O_2 occurs by reduction but the bulk of the O_2 in solution remains unreduced. Even bubbling with 1% O_2 does not alter the electron requirements for reduction (A. M. Rauth, personal communication) neither does the addition of catalase or bubbling N_2 through alkaline dithionite which decreases oxygen contamination drastically. Nevertheless, it is obvious that damage to ØX174 DNA is strongly dependent on the rate of reduction of the drugs; the slower the reduction rate the greater the damage. The one-electron nitro radical anion of 5-nitroimidazoles decays with second order kinetics whereas the decay of 2-nitroimidazole anions shows predominantly first order kinetics [21].

With 5-nitroimidazoles at high reduction rates this would tend to favour disproportionation to the parent drug and the nitroso derivative

$$2\text{R—NO}_2^{\cdot} \longrightarrow \text{R—NO}_2 + \text{R—NO} + \text{H}_2\text{O}$$

A similar reaction might perhaps occur for 2-nitroimidazoles, preceded, however, by a first order protonation [21]. The resulting nitroso compounds are further reduced to the corresponding hydroxylamine. The hydroxylamine derivative of metronidazole is inactive as a cytocidal agent [23]. The one of misonidazole was obtained by irradiation in the isopropanol/N_2O system at pH 4.0 at a reduction rate of 2%/hr. At this pH the hydroxylamine derivative is relatively stable [24, 25]. Incubation with single or double stranded ØX174 DNA shows no inactivation (unpublished results and in ref. 17). Further, electron values for reduction decrease from about 4 with 2-nitroimidazoles to between 3 and 4 with 5-nitroimidazoles [11] and also varies in the presence of DNA suggesting the nitroso derivative as a possible candidate for the short-lived damaging agent. However, the formation of the nitroso derivative would be favoured by disproportionation which would dominate at high reduction rates. If this occurred increased damage would be expected as the reduction rate increases but this is clearly not the case.

Nitroso formation would also be favoured at acid pH because the disproportionation reaction requires two protons. Although damage is increased ([19] and this study), this could equally be due to protonation of the nitro radical anion i.e. R—NO_2H.

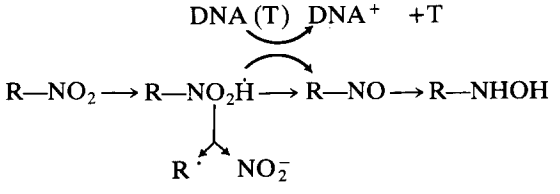
The protonated radical anion is more stable [21, 22] and its decomposition to form nitrite and the imidazole radical, especially with 5-nitroimidazoles would be less likely explaining the decreased nitrite yields at acid pH. This would leave a greater number of R—NO_2H molecules for further reduction explaining the increased N values at high pH for misonidazole and other typical 2-nitroimidazoles. It has often been suggested that the damage observed in DNA during reduction of nitroimidazoles is due to the action of a nitro-radical [11, 26, 27].

We have shown recently [28] that the damage produced to double-stranded ØX174 DNA by reduced nitroimidazole is not repaired by the excision repair pathway in *E. coli* which would be expected for adducts [28]. Moreover, nitroimidazoles in general have little effect on the SOS repair pathway as measured by sister chromatid exchange [29, 30]. This suggests that strand breakage of DNA is caused by a mechanism which does not involve binding of reduced drug to form stable adducts. That the binding of reduced misonidazole to *E. coli* DNA is several orders of magnitude less than the damage produced (strand breakage leading to the specific

release of thymidine) and is not related to the % A+T composition of the DNA [10] supports the above contention.

We suggest the protonated one-electron nitro radical anion as a more plausible candidate for the short-lived damaging agent as its formation at low reduction rates would favour reaction with DNA and is compatible with the general scheme proposed by Brown [31].

The mechanism of such a reaction is speculative but we envisage $R—NO_2\dot{H}$ acting as an electron acceptor (or $R—NO_2^{\cdot-}$ acting as an electron and proton acceptor) from DNA, resulting in the observed decrease in N values in the presence of the target, presumably in the region of thymidine residues causing strand breaks and the release of thymidine (T) as shown below:



The model which is analogous to the reaction of a nitrobenzene radical anion with alkylhalides [32] would also predict that damage (strand breaks) to DNAs of high % A+T content would decrease the value of N as shown in ref. 10. The damage produced does not require binding of reduced drug intermediates to form DNA adducts, nor does it involve oxygen other than futile cycling at relatively high O_2 levels. Furthermore, the increased DNA damage with decreasing reduction rate, predicted by the model (due to the disproportionation reaction in competition with reaction with the DNA) is too small compared with the experiments (shown in Fig. 2). This probably means that the damaged DNA molecules, before becoming inactivated, have to undergo a further rate-dependent reaction with a reduction product in competition with a restoration of the biological activity of the DNA molecules.

Rauth and co-workers [18] have found that less O_2 is required to inhibit metronidazole reduction than misonidazole measured at a dose rate of 60 Gy/hr as is also found in CHO cells. These observations may explain why metronidazole is less toxic than misonidazole in hypoxic cells. Under the same conditions of hypoxia, although the reaction of metronidazole nitro radicals anions towards DNA is more efficient than misonidazole, nevertheless, metronidazole radical anions will be present at a much lower concentration because less O_2 is required to inhibit metronidazole reduction. Therefore the overall effect will be more damage with misonidazole than with metronidazole.

The decomposition to form nitrite is negligible in 2-nitroimidazoles, nitrofurans and nitrobenzene derivatives but assumes an important pathway for 5-nitroimidazoles where over 30% NO_2^- is produced at neutral and alkaline pH [11, 26, 27].

Finally, we would suggest that the correlation of DNA damage with reduction potential (E_7^1) may reflect either the relative activity of the protonated radical or its relative stability rather than the rate of reduction as it is found for correlations of opposite slope [22].

Acknowledgements—We thank the Cancer Research Campaign, the Medical Research Council of the UK and the Koningin Wilhelmina Fonds of the Netherlands for Financial support which is gratefully acknowledged. And Miss J. Worrell for her excellent secretarial assistance.

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