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INDUCTION OF DNA STRAND BREAKS BY REDUCED NITROIMIDAZOLES

IMPLICATIONS FOR DNA BASE DAMAGE

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Abstract—Radiation-reduced 2-nitroimidazoles (misonidazole, RSU-1137, Ro.03-8799 and Ro.03-8800) incubated in air with plasmid DNA (pH 7.0, 310K) induce DNA strand breakage, as revealed following subsequent heat or alkali treatment. Only RSU-1137 resulted in the binding of a [2-^{14}C] fragment and significant yields of heat-labile strand breaks (>20% loss of type-I DNA after 48 hr incubation). RSU-1137 was shown to be >6 times more effective than misonidazole at producing alkali-labile breaks. In fact, the efficiency of alkali-induced strand break production is in the order: misonidazole < Ro.03-8799 ≈ Ro.03-8800 < RSU-1137. Reaction of these reduced 2-nitroimidazoles with 2′-deoxyguanosine (dG) also results in the formation of a common glyoxal-dG product, with its yield and rate of production being dependent upon the 2-nitroimidazole used. It has been demonstrated that these variations are influenced by the *N*-1 side-chain of the 2-nitroimidazole. Product yields are ~5–6 times greater with misonidazole than with RSU-1137. From the evidence presented, it is apparent that formation of glyoxal (or a glyoxal-like product) is not responsible for the DNA strand breakage seen. It is inferred that these breaks are induced by a nitro-reduction product(s) which remains unidentified.

Nitroimidazoles are currently undergoing clinical trials as hypoxic cell radiosensitisers for the treatment of human tumours [1–3]. These compounds may also have clinical application in chemotherapy since nitroimidazoles have been shown to be differentially cytotoxic towards hypoxic mammalian cells [1, 4–8]. This preferential toxicity is likely to be related to the ability of the nitro-compounds to undergo bio-reduction, under anaerobic conditions, leading to the formation of reactive species which may interact with critical cellular targets [9, 10].

Previous studies have shown that reductively-activated nitroimidazoles bind to various intracellular targets including DNA, RNA, proteins and non-protein thiols [10–17]. DNA has been implicated as a major cellular target and studies on the mode of action of the drugs have indicated that the degree of binding of the reduced nitroimidazole to DNA *in vitro* depends upon the nitro-compound used [12, 18, 19]. With the *aziridine*-containing nitroimidazole, RSU-1069 [NSC 347503, 1-(2-nitro-1-imidazolyl)-3-(1-aziridinyl)-2-propanol], such an interaction is accompanied by production of DNA strand-breakage at pH 7 [18, 20]. This phenomenon is not observed using either misonidazole or RSU-1137 [3-(2-hydroxyethyl)amino-1-(2-nitro-1-imidazolyl)-2-propanol, structures in Fig. 1], the aziridine ring-opened hydrolysis product of RSU-1069. At the chemical level, reduced misonidazole [21] and other reduced 2-nitroimidazoles [22] bind to guanine to yield a covalent product, which in the case of misonidazole, resembles that of a glyoxal-guanine adduct [23].

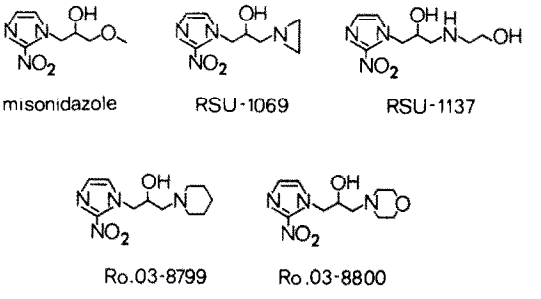

Fig. 1. Structures of the 2-nitroimidazoles used in this study.

The aim of the present study was to investigate further the interactions of radiation-reduced misonidazole, RSU-1137 and other 2-nitroimidazoles with plasmid DNA in order to shed light on the nature of the reduction product(s) which leads to differential binding to and strand breakage of DNA. Further, the interaction of these reduced 2-nitroimidazoles with 2′-deoxyguanosine (dG) has been studied to investigate structural effects upon product distribution.

MATERIALS AND METHODS

Synthesis of RSU-1069, 8799 and 8800. RSU-1069 was prepared using the method of Adams *et al.* [24]. Ro.03-8799 and Ro.03-8800 (3-piperidino- and 3-morpholino-1-(2-nitro-1-imidazolyl)-2-propanol,

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"8799" and "8800", respectively) were synthesised using the procedure of Smithen *et al.* [25].

Synthesis of RSU-1137. RSU-1137 was prepared by condensation of 1-(2,3-epoxypropyl)-2-nitroimidazole [26] with 2-aminoethanol in refluxing ethanol solution. Removal of solvent *in vacuo* at completion of the reaction and crystallisation from ethanol afforded pale-yellow prisms, m.p. 414–415 K; yield 87%. This material was homogeneous by thin-layer chromatography and had elemental analysis (C,H,N) and spectroscopic parameters (i.r., u.v., ^{1}H NMR and mass spectrum [E–I: m/e 231 $(M+1)^+$, 184 $(M^+ - NO_2)$ base peak]) consistent with the structure shown.

Synthesis of [2-^{14}C]8799 *and* 8800. [2-^{14}C]-Labelled Ro.03-8799 and Ro.03-8800 (labelled at imidazole ring *C*-2 and each of specific activity 25.2 MBq/mmol) were prepared by a procedure analogous to that previously reported [18] for [2-^{14}C]RSU-1069. Thus, condensation of the [2-^{14}C]-labelled 1-(2,3-epoxypropyl) derivative [18] with piperidine or morpholine in refluxing ethanol solution afforded, after concentration *in vacuo* and crystallisation from ethanol, the propanolamines as pale-yellow crystals. The products were homogeneous by thin-layer chromatography and indistinguishable from the unlabelled compounds by u.v. and i.r. spectrophotometry. [2-^{14}C]8799 had m.p. 383–384 K (lit.: 383–385 K [25]); [2-^{14}C]8800 had m.p. 384.5–385 K (lit.: 385–386 K [25]).

Other chemicals. All reagents with the exception of analytical grade material for phosphate buffers (BDH) were from Sigma. Glyoxal (ethanedial, 40% w/w solution in water) was obtained from Aldrich and used as supplied.

Radiation-reduction of 2-nitroimidazoles Radiation-reduced drugs were generated by irradiation of N_2-saturated aqueous solutions of the compounds (2–11 mmol/dm^3) containing 0.2 mol/dm^3 sodium formate, buffered at pH 7.0 with 0.1 mol/dm^3 phosphate, using a 4.3 MeV linear accelerator as detailed previously [18]. In all experiments the solutions were irradiated to effect ≥90% conversion of the 2-nitroimidazole (4.0 ± 0.3 reducing equivalents per molecule) to reduced product(s) [18], as determined spectrophotometrically at 325 nm using a Beckman DU-8B spectrophotometer.

Reaction of radiation-reduced 2-nitroimidazoles with 2'-deoxyguanosine (dG). Unless stated otherwise, solutions containing the radiation-reduced drugs were diluted with aqueous solutions of dG under aerobic conditions at pH 7.0 to generally give a final concentration of 8.0 mmol/dm^3 reduced 2-nitroimidazole and 1.0 mmol/dm^3 dG. The reaction mixtures were incubated at 310 K and at given time intervals, aliquots were removed and monitored using a Beckman 344 liquid chromatography system (5 μm Spherisorb-ODS column, Hichrom Ltd., Reading, U.K.; mobile phase: 10 mmol/dm^3 aqueous KH_2PO_4 containing 10% v/v methanol, pH 6.0; flow rate: 1 cm^3/min. Reaction components monitored at 260 nm).

The adduct from reaction of glyoxal with dG was prepared as detailed previously [23] and analysed using HPLC as described above.

Reaction of radiation-reduced 2-nitroimidazoles with plasmid DNA. The plasmid pBR322 was maintained in *E. coli* HB101 and extracted using conventional methods [27]. DNA concentrations were determined by spectrophotometry. Parent or radiation-reduced drug solutions (9–11 mmol/dm^3) were diluted with a solution of plasmid DNA and incubated at 310 K under aerobic conditions. Final concentrations were 180 μg/cm^3 plasmid DNA and 6.5–8 mmol/dm^3 reduced drug.

Following exposure to the drug for given time intervals, plasmid DNA was assayed for strand breakage after a post-incubation alkali or heat-shock treatment. Neither of these treatments results in degradation of type-I (closed-circular) DNA. The alkali treatment involved the addition of 0.5 mol/dm^3 aqueous NaOH to the reaction mixture (final pH 12.5), followed by 10 min incubation at 293 K. Heat-shock involved precipitation of the plasmid DNA from the reaction mixture by addition of 2 mol/dm^3 sodium acetate (0.1 volume) followed by ice-cold ethanol. Samples were then spun at 12,000 *g* for 4 min, the supernatant removed and the pelleted DNA resuspended in water. The precipitation process was repeated and the pelleted DNA finally resuspended in 35 mm^3 water. The plasmid DNA was heated in a water bath at 363 K for 1 min and then immediately placed on ice.

In order to assess DNA strand breakage induced by free glyoxal, plasmid DNA (180 μg/cm^3) was incubated in aqueous solution with glyoxal (0.1 mmol/dm^3) for 24–30 hr at 310 K, followed by an alkali treatment.

DNA strand breakage [type-I→type-II (open-circular)] was assayed using agarose (0.8%) gel electrophoresis and densitometry as detailed previously [18].

Binding to DNA in vitro. The percentage binding of radiation-reduced [2-^{14}C]-labelled 8800 and 8799 (1 mmol/dm^3) to calf thymus DNA *in vitro* was determined as described previously [18].

RESULTS

Reaction of reduced 2-nitroimidazoles with 2'-deoxyguanosine (dG)

The interaction of radiation-reduced RSU-1069, RSU-1137, 8800, 8799 and misonidazole with dG results in, as identified by HPLC, a product whose retention time (11.0 min) is independent of the 2-nitroimidazole used. The product has an optical absorption spectrum which corresponds with that of a glyoxal-guanine adduct identified previously [21, 23] and with that of the synthesised adduct of glyoxal and guanosine. The retention time for the latter corresponded with that for the product generated on interaction of dG with the reduced 2-nitroimidazoles. The optical absorption spectra and HPLC chromatogram are shown in Fig. 2. The yield (Table 1) and timecourse of production of the glyoxal-dG adduct is, however, dependent upon the 2-nitroimidazole used, as shown in Fig. 3. In solutions containing dG (1 mmol/dm^3) and reduced nitroimidazole (8 mmol/dm^3), the rate of formation of the glyoxal-dG product is first-order; the half-lives for the reaction with reduced misonidazole and RSU-1137 were determined to be (6 ± 1) and (2 ± 0.5) hr,

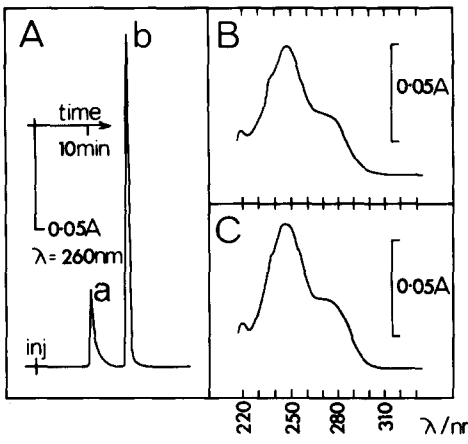


Fig. 2. A: HPLC chromatogram (conditions given in text) of radiation-reduced misonidazole after incubation with dG (pH 7.0, 310 K) for 48 hr; peak a = glyoxal-dG product and peak b = 2′-deoxyguanosine. B and C: optical absorption spectra of the glyoxal-dG product resulting from interaction of reduced misonidazole with dG (peak a in panel A) and of the synthesised glyoxal-dG adduct, respectively.

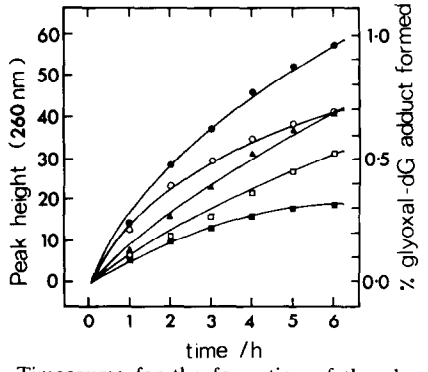


Fig. 3. Timecourse for the formation of the glyoxal-dG product, as assayed using HPLC, on interaction of dG (1 mmol/dm^3) with radiation-reduced 2-nitroimidazoles (8 mmol/dm^3) at pH 7; ● misonidazole, ○ RSU-1069, ▲ Ro.03-8799, □ Ro.03-8800, ■ RSU-1137. Each point represents the mean value (±5%) from at least two determinations.

respectively. Further, the yield of this product on completion of the reaction is ~6 times greater with misonidazole than with RSU-1137. From the yield and rate of loss of dG, as monitored by HPLC, it is apparent that the loss of dG is predominantly via formation of the glyoxal-dG adduct.

Since formation of this product is indicative of glyoxal participation, it is inferred that variations in the yield and timecourse for the formation of the glyoxal-dG adduct may, with the exception of misonidazole, be due to competing reactivity of the formed glyoxal with the basic side-chain of the 1-(aminoalkyl)-substituted 2-nitroimidazoles (see Table 1 for pK_a values). In order to test whether amines can protect against the formation of glyoxal-guanine product(s), the following experiment was performed. The addition of a secondary aliphatic amine [2-(methylamino)ethanol, 10 mmol/dm^3] to the reaction mixture containing reduced misonidazole (8 mmol/dm^3) and dG (1 mmol/dm^3) results in ~95% inhibition of product formation, as determined by HPLC. The amine side-chains of RSU-1137, RSU-1069, 8800 and 8799 may therefore act as reactive sites for glyoxal in competition with dG.

No major reaction products were identified under our HPLC conditions when radiation-reduced misonidazole and RSU-1137 were exposed to either pyrimidines (2′-deoxycytidine and thymidine) or the purine (2′-deoxyadenosine) for 30 hr at 310 K.

Binding of reduced 2-nitroimidazoles to DNA

The percentage binding ratios for radiation-reduced [2-^{14}C]-labelled RSU-1137 [18], 8800, 8799 and misonidazole [18] to calf thymus DNA after 6 hr exposure, or 1 hr exposure for RSU-1069, are presented in Table 1. RSU-1137, as a reduced species, binds within 1 hr [18] and to a greater extent than other reduced 2-nitroimidazoles. The binding ratio of RSU-1137 is at least one order of magnitude greater than that of the other nitroimidazoles examined, with the exception of RSU-1069, for which the binding ratio increases with time to a "plateau" value (~6 molecules bound per 100 nucleotides) [18].

Induction of strand breakage in plasmid DNA by reduced 2-nitroimidazoles

Radiation-reduced RSU-1137, 8800, 8799 and misonidazole do not cause strand breakage of plasmid DNA at pH 7.0, even after incubation for 72 hr

Table 1. The maximal yield of glyoxal-dG adduct and the percentage [2-^{14}C] binding to DNA on reduction of 2-nitroimidazoles

Nitro-compound	Yield of glyoxal-dG* (μmol/dm^3)	% [2-^{14}C] binding†	pK_a
Misonidazole	21.4 (2.0%)	<0.04‡	—
RSU-1137	3.6 (0.3%)	2.8‡	7.95
RSU-1069	8.3 (0.8%)	2.7‡	6.04§
Ro.03-8800	n.d.	0.2	6.15‖
Ro.03-8799	n.d.	0.08	8.71‖

* Reaction conditions given in text.
† Binding ratios expressed as molecules bound per 100 nucleotides after 6 hr incubation (or 1 hr with RSU-1069).
‡ From ref. [18].
§ From ref. [32].
‖ From ref. [33].

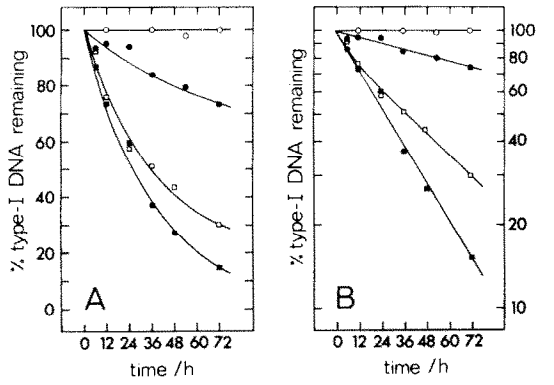


Fig. 4. A: Timecourse for the induction of strand breakage of plasmid DNA, expressed as the percentage of type-I DNA remaining, on interaction with reduced 2-nitroimidazoles followed by a heat or alkali treatment. B: Semi-log representation of the data in (A). Misonidazole (○, ●) and RSU-1137 (□, ■) where open and closed symbols represent heat and alkali treatment, respectively. Each point represents the mean value from at least three determinations with estimated errors of ±10%. Data are normalised for the type-II (open-circular) DNA present prior to incubation.

at 310 K. However, a post-incubation heat or alkali treatment of the DNA following such exposure to radiation-reduced 2-nitroimidazoles results in the formation of strand breakage over the time-course shown in Fig. 4 for RSU-1137 and misonidazole. The yields of DNA strand breakage following heat and alkali treatment (expressed as a percentage loss of type-I (closed-circular) DNA) produced by the reduced compounds after 48 hr are presented in Table 2.

In the case of heat-labile sites, only reduced RSU-1137 induces considerable damage (≥20%) of the DNA. The first half-life for strand breakage by RSU-1137 upon heat treatment (Fig. 4) was determined to be ~(37 ± 3) hr. For alkali-labile sites (Table 2), the efficiency of strand break production is in the order RSU-1137 ≫ 8800 ≈ 8799 > misonidazole. As shown in Fig. 4 for RSU-1137 and misonidazole, the rate of loss of type-I DNA with time is exponential, from which it is estimated that the half-life for strand break formation is (26 ± 2) and ≫72 hr using reduced RSU-1137 and misonidazole, respectively. From the slopes of the curves it is estimated that reduced RSU-1137 is ~6 times more efficient at producing alkali-labile sites than misonidazole.

Table 2. The percentage of type-I DNA remaining after 6 and 48 hr incubation with reduced 2-nitroimidazoles, followed by heat or alkali treatment

	% type-I DNA remaining			
	heat		alkali	
Nitro-compound	6 hr	48 hr	6 hr	48 hr
Misonidazole	100	100	93	80
RSU-1137	92	43	87	27
Ro.03-8800	n.d.	84	92	67
Ro.03-8799	87	85	88	60

Since glyoxal or a glyoxal-like product, which on reaction with dG yields the glyoxal-dG adduct, is produced on reduction of the 2-nitroimidazoles (see above), the ability of free glyoxal (0.1 mmol/dm^3) to induce DNA strand breaks was examined. After 24–30 hr incubation at 310 K, glyoxal does not induce either direct or alkali-labile strand breaks, as evidenced by the stability of the type-I DNA (i.e. <10% degradation).

In order to gain information on the stability of the species required for strand breakage, radiation-reduced RSU-1137 and misonidazole were maintained at 310 K for 48 hr prior to addition of the DNA and incubation for a further 6 hr. The yields of heat- and alkali-labile sites are <10% (loss of type-I DNA), from which it is inferred either that the reactive species is unstable or that the amount of damage induced is equivalent to that observed after 6 hr as shown in Fig. 4. Incubation of reduced misonidazole or RSU-1137 with DNA in the presence of dG (1 mmol/dm^3) for 48 hr results in >90% protection, as witnessed from the decrease in heat- and alkali-labile sites (<10% of control within 48 hr).

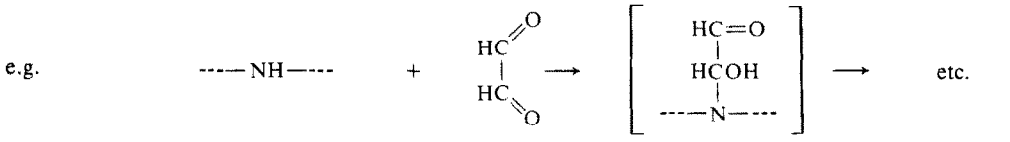
Since the hydroxylamines produced on reduction of 2-nitroimidazoles [28, 30] are unstable ($t_{1/2}$ < 5 min) even under anaerobic conditions, the following experiment was undertaken to assess the involvement of a hydroxylamine species and its possible conversion with oxygen to yield potentially toxic products. After radiation reduction of RSU-1137 or misonidazole, the reduced metabolites were either incubated with DNA in air for 48 hr or kept anaerobic for ~1 hr after reduction, prior to incubation with DNA in air for 48 hr. The yield of alkali-labile sites is independent of the 1 hr anoxic period prior to incubation with DNA.

DISCUSSION

The results of this study add further support to the idea that a product(s) resulting from the reduction of nitroimidazoles may play a role in the modification of cellular constituents under hypoxic conditions. The efficiency with which nitroimidazoles act as hypoxia-selective cytotoxic agents may be reflected in the nature of the products formed. As previously highlighted [18], the binding efficiency of reduced nitroimidazoles to DNA ([2-^{14}C] fragment) may be influenced by the nature of the *N*-1 side-chain. Assuming that the pK_a of the side-chains of radiation-reduced products of 8800 and 8799 are essentially unaltered, the lack of binding shown, with the exception of reduced RSU-1137 (Table 1), is not consistent with the state of protonation of the base function influencing the ability of the reduced product(s) to bind to DNA, such as via a facilitated electrostatic interaction. Whether the piperidino- and morpholino-base groups of 8799 and 8800 are sufficiently bulky to sterically influence such interaction is, as yet not known. RSU-1069, as parent and reduced compound, binds to DNA [18], however the aziridine moiety plays a dominant role in its interactions thereby complicating any direct comparison with the other 2-nitroimidazoles.

A common product following interaction of reduced 2-nitroimidazoles with dG is identified as a glyoxal-guanine adduct which has previously been reported to be formed following chemical reduction of misonidazole [17, 21, 31]. The formation of such an adduct with DNA cannot result in the incorporation of a [2-^{14}C] fragment into the DNA but represents potential modification of DNA involving non-[2-^{14}C] fragments.

The yield and rate of production of the glyoxal-dG product is dependent upon the nature of the nitroimidazole basic side-chain, as implied from the influence of 2-(methylamino)ethanol. From the protective effect seen with misonidazole, it is proposed that an amine-containing side-chain may interact with the formed glyoxal (or a product which is glyoxal-like in nature) in competition with dG:

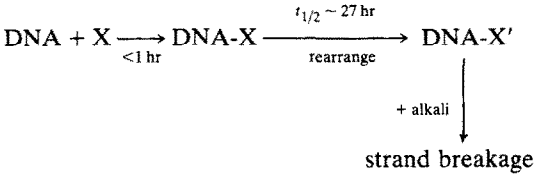


Such a competition is consistent with the observed variations in rate of formation and yield of the glyoxal-dG product with the 2-nitroimidazoles. Indeed, the yield of the product is ~5–6 times greater with misonidazole as compared to that with RSU-1137. If these variations are reflected in their DNA interactions, then reduced misonidazole should be more efficient than reduced RSU-1137 in producing DNA base damage via interaction of a product with glyoxal-like properties. Reduced RSU-1137 is however more efficient than reduced misonidazole at producing alkali-induced strand breaks (see later), therefore it is proposed that interaction of a glyoxal-like reduction product with DNA does not lead to strand breakage, even under alkaline conditions. This proposal is supported by both the observed lack of strand breakage induced by free glyoxal, even under alkaline conditions, and the known instability of the glyoxal-guanine adduct (resulting in regeneration of guanine) at pH >9 [21].

With the exception of reduced RSU-1069, which operates by a different mechanism [18, 20, 22], the nitroimidazoles upon radiation-reduction do not cause strand breakage of DNA at pH 7.0, even though reduced RSU-1137 shows significant binding to DNA within 1 hr [18]. With all the reduced 2-nitroimidazoles examined, strand breakage of DNA is, however, revealed upon subsequent alkali treatment, indicating the presence of base-damaged sites. After incubation of reduced nitroimidazoles with DNA, only RSU-1137 results in the formation of significant yields of heat-labile breaks (>15% loss of type-I DNA after 48 hr incubation). Based upon the kinetics of alkali-induced strand break formation, RSU-1137 is more than ~6 times as effective as misonidazole at producing "indirect" strand breakage. As discussed above, this strand breakage does not reflect formation of a glyoxal-guanine adduct upon the DNA. From these results it is therefore inferred that a product(s) of reduction, as yet unidentified, is responsible for the observed cleavage damage to DNA. Indeed, with RSU-1137 it is inferred that a product(s) is formed which is not produced with misonidazole. The protection against strand breakage afforded by dG with reduced misonidazole and RSU-1137, suggests that the reduction product(s) which induces such breakage is efficiently removed by dG and, further, implies that guanine is a potential site for interaction upon the DNA.

The possible involvement of products resulting from the interaction of O_2 with a hydroxylamine species (e.g. arising by addition of aerated DNA to reduced drug) is not compatible with the observation that the yield, after 48 hr, of alkali-induced DNA strand breaks with RSU-1137 and misonidazole is not modified after maintaining the (anaerobically) reduced drugs for 1 hr under N_2 prior to addition of the DNA. This time is sufficiently long, compared to the reported lifetimes of analogous 2-hydroxylaminoimidazoles [28, 30], to make this species no longer significant. Furthermore, under the normal conditions used for preparation of the reduced drugs, the irradiation period is sufficiently long compared to the lifetime of such species that the majority of the hydroxylamines formed will have decomposed under anaerobic conditions prior to the addition of the aerobic DNA. Similarly, the short-lived nitroimidazole radical-anion species formed ($t_{1/2}$ <10 sec [34]) will not be present at the time of addition of the DNA. Whether the presence of air modifies the more stable reduction products of nitroimidazoles is not known.

Since only reduced [2-^{14}C]RSU-1137 binds significantly to DNA and causes significant strand breakage within 48 hr, the resulting bound product may be the precursor to the alkali- and heat-labile breaks. However, the timescale over which the two processes occur are separated by 1–2 orders of magnitude. From this difference it is implied that the initial bound product would have to undergo a rearrangement prior to strand breakage and that this rearrangement would be the rate-determining step, i.e.



Since the other reduced nitroimidazoles also cause alkali-induced strand breaks, but to a lesser degree, it is suggested that further damaged DNA sites are produced by an unidentified reduction product and that the process does not necessarily involve incorporation of a [2-^{14}C] fragment and cannot be due to glyoxal or a glyoxal-like product.

In conclusion, products of nitro-reduction other than glyoxal (or a glyoxal-like compound) are responsible for DNA strand breakage. The induction of these separate events may be relevant to the mechanism of the cytotoxicity shown by 2-nitroimidazoles towards hypoxic mammalian cells [1], particularly as high concentrations of a glyoxal (>1 mmol/dm^3) have been shown to result in DNA-protein cross-links [35]. The information obtained in the present study may find application in the design of improved hypoxia-selective cytotoxic agents since the induction of alkali-labile DNA strand breaks by nitro-compounds may be used as a cellular marker of bio-reduction.

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