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REDUCTION OF NITROIMIDAZOLES *IN VITRO* AND DNA DAMAGE

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Nitroimidazoles were first used clinically 25 years ago with the introduction of metronidazole for the treatment of vaginal infections caused by the protozoan *Trichomonas vaginalis* [1]. Since then nitroimidazoles have found widespread use in treatment of infections by facultative and obligate anaerobic bacteria, anaerobic protozoa and, in the last 10 years, as radiosensitizers for the treatment of hypoxic tumours. Current clinical uses of these drugs are listed in Table 1, and representative structures are given in ref. 2. Prior to 1960 vaginal trichomoniasis was widespread with an incidence ranging from 5 to 70% [3] and the disease was commonly treated with vinegar douches or methyl violet-impregnated tampons. Such treatment was largely ineffective because the parasite, once sheltered within the confines of Skene's or Bartholin's glands, created reservoirs to reinfect the vagina being thus inaccessible to topical agents. Metronidazole was an important chemotherapeutic advance because it could be used systemically and was clinically very effective. Since 1960 other nitroimidazoles have appeared for the treatment of infectious disease. Of these tinidazole and ornidazole are in widespread clinical use and others include secnidazole, nimorazole, carnidazole and the 2-nitroimidazole benznidazole, which is one of the most effective drugs for the treatment of *Trypanosoma cruzi* infections (Chagas disease). Secnidazole has the longest serum half-life of any clinically useful nitroimidazole (17 hr), compared with 12–13 hr for the tinidazole and satranidazole, and 8 hr for metronidazole. Satranidazole is a recent and novel 5-nitroimidazole possessing a methylsulphonylimidazolidinone at C2 of the imidazole ring. Unlike other 5-nitroimidazoles the bond at C2 is C–N rather than a carbon–carbon link and this feature may be relevant to its activity which is reported as being superior to metronidazole and other 5-nitroimidazoles for amoebiasis [4, 5], trichomoniasis [6] and anaerobic infections *in vitro* and in the mouse [7].

Although several nitro-compounds were known to sensitize cells to ionizing radiation, including para-nitroacetophenone and nitrofurans, it was the discovery of metronidazole as radiosensitizer which boosted research in this field of radiobiology [8]. Its ability to selectively radiosensitize hypoxic cells both *in vitro* and *in vivo* [9, 10] coupled with its known clinical use rapidly led to radiotherapy trials [11]. However, since the principles of electron affinity first proposed by Adams and Dewey [12] predicted better radiosensitization with more electron affinity, attention soon turned to 2-nitroimidazoles which showed better radiosensitization properties in hypoxia [13]. These drugs included misonidazole and its desmethyl derivatives, SR-2508, Ro 03-8799 and RSU-1069. Although misonidazole showed good radiosensitizing properties *in vitro* and *in vivo* its use in humans is limited by neurotoxicity at doses required to achieve good tumour penetration. The desmethyl analogue also shows some peripheral neuropathy at the maximum tolerated dose of misonidazole (12 g/m^2) although no central effects, and is unlikely to show any substantial advantages over misonidazole. The compound SR-2508 is also a misonidazole analogue but is more promising. It shows better tumour

Table 1. Clinical uses of nitroimidazole drugs in humans

Disease	Causative organism	Drug type
Trichomoniasis	*Trichomonas vaginalis* (P)	5-NI
Non-specific vaginitis	*Gardnerella* (= *Haemophilus*) *vaginalis* (B)	
Intestinal amoebiasis	*Entamoeba histolytica* (P)	
Intestinal amoebiasis	*Giardia intestinalis* (= *lamblia*) (P)	
Intestinal amoebiasis	*Balantidium* sp (P)	
Post-operative sepsis	*Bacteroides* sp (B)	
Post-operative sepsis	*Clostridium* sp (B)	
Leishmaniasis	*Leishmania mexicana* (P)	
Vincent's stomatitis	Many, including *Bacteroides* (B) and fusiforms (B)	
Crohn's disease	Unknown	
Hypoxic cell tumours	—	2-NI

P, protozoan; B, bacterium; 5-NI, 5-nitroimidazole derivatives; 2-NI, 2-nitroimidazole derivatives.

penetration and can be given at higher doses without neurological side effects. Ro 03-8799 is again an analogue of misonidazole but has a higher radiosensitization efficiency and better tumour uptake. A recently developed compound, RSU-1069 is a 2-nitroimidazole, but also carries a side chain containing an alkylating aziridine ring. This drug shows a 10-fold increase in efficiency compared with misonidazole and although more toxic could be the basis of a new type of 2-nitroimidazole radiosensitizer which deserves further investigation. All these drugs, beside showing radiosensitizing properties are selectively cytotoxic to hypoxic cells to some extent and also show properties of chemosensitization, i.e. they enhance the action of other cytotoxic drugs, particularly cyclophosphamide, nitrosoureas and alkylating agents [14, 15].

The following discussion will centre on the cytotoxic effect of nitroimidazole drugs with particular reference to reduction of the nitro group and DNA damage.

REDUCTION AND ENTRY INTO THE CELL

Nitroimidazoles are preferentially cytotoxic to hypoxic cells [16, 17]. Toxicity to aerobic cells also occurs but only after longer exposure times or with drug concentrations 5–50 times greater per unit time [17, 18]. In both hypoxic mammalian cells, protozoa and anaerobic bacteria, the effect is only produced after reduction of the nitro group [19–22] and, depending on the reduction potential of the drug, the rate of reduction varies over three orders of magnitude [23, 24]. In mammalian cells reduction of the nitro group is mediated by NADH and flavoproteins including xanthine oxidase, NADPH cyt P450 reductase and NADPH cyt *c* reductase all of which can reduce the more electron affinic 2-nitroimidazoles [25], but with the low electron affinic 5-nitroimidazoles the electron source in anaerobes is the pyruvate dehydrogenase ferredoxin oxidoreductase complex and ferredoxin itself [21, 26, 27]. The rate of intracellular reduction is important because it determines the rate of drug uptake into the cell via a concentration gradient.

In anaerobic microorganisms the ferredoxin-linked pyruvate dehydrogenase system converts pyruvate or pyruvate and phosphate to acetyl CoA or acetyl phosphate and liberates CO_2 and H_2. Low redox potential 5-nitroimidazoles inhibit H_2 evolution as a primary event, but the system recovers once all the drug is reduced [28, 29]. This observation led to the tacit assumption that a reduction product of the drug was responsible for cell killing.

NITROIMIDAZOLES DAMAGE DNA

It was already known that other nitroheterocyclic drugs, nitrofurans for example, damage DNA [30, 31], and thus it was not surprising that metronidazole inhibited uptake of radiolabelled thymidine into the DNA of *T. vaginalis* [32] nor that other 5-nitroimidazole drugs including tinidazole not only inhibited DNA synthesis, but caused degradation of existing DNA [33]. Subsequently it was shown that cytotoxicity of misonidazole and DNA damage in hypoxic mammalian cells were related [34], and Olive showed that both metronidazole and misonidazole caused DNA damage under hypoxia in mammalian cells and that there was a correlation between the inhibition of DNA synthesis or mutagenicity and the reduction potential of nitroheterocycles [24]. The identification of DNA as a target for reduced nitroimidazoles in clinically relevant organisms and cell culture thus enabled detailed *in vitro* studies to be carried out on the drug–target interaction.

The first of these studies showed that metronidazole reduced by sodium dithionite in the presence of DNA induced DNA damage by a non-intercalative, non-crosslinking mechanism typical of strand breakage and helix destabilization [35]. Dithionite itself, however, produced DNA strand breaks making unambiguous determination difficult [36]. In order to study the interaction of reduced nitroimidazoles with DNA or to identify reduction products several approaches have been adopted to reduce selectively the nitro group. These include electrolytic reduction at constant potential under N_2 [34, 37–43], reduction with zinc and ammonium or calcium chloride in air [44–46], by radiolysis [47–49], using xanthine oxidase [50–52], or by using intestinal microflora or mammalian cells in tissue culture [53, 54].

The first indication that the action of reduced nitroimidazoles in causing DNA damage may not be random came from the observation that helix destabilization and strand breakage were related to the base composition of DNA [43] in that DNAs of high % A + T content were more susceptible to damage than those of low % A + T. Subsequently, damage was found to be associated with the specific release of a mixture of thymine and thymidine phosphates [38, 43]. This effect was not associated with any significant binding to DNA of misonidazole labelled in the 2-position of the imidazole ring [38]. Damage appears to be specific irrespective of the technique used to measure the damage and is maximal with poly(d[AT]) polymers and absent with poly(d[GC]) [43, 55]. Quantitative structure–activity relations of the Hansch type indicate that those nitroimidazoles of relatively low reduction potential are more effective in damaging DNA and releasing thymidine derivatives than those of higher potential [38, 56]. Recent work using a bacteriophage (ϕX174) transfection assay has not only corroborated the above results, but shown that the correlation equations apply to biologically relevant DNA damage.* However, the correlation between reduction potential and drug damaging activity has the opposite slope of that found for hypoxic cytotoxicity and radiosensitization of 2- and 5-nitroimidazoles ([57, 58] for recent reviews). A possible explanation of this anomaly will be discussed later.

Binding of reduced labelled misonidazole and other nitroimidazoles to DNA certainly occurs, but to date there is no evidence that binding correlates with damage. A recent report [59] suggests guanine as a target for 5-nitroimidazoles based on electrolytic

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* Zahoor *et al.* submitted to *Int. J. radiat. Oncol. Biol. Phys.*

reduction of the drugs in the presence of end-labelled DNA. This approach also showed cleavage at T residues, but the method detects only base modifications which are capable of being displaced by piperidine. Further, since the technique involves precipitation and redissolving of DNA after reaction with the drug only relatively intact DNA will be recovered as highly fragmented or low molecular weight DNA will not precipitate. The method thus preferentially selects modified or adduct/altered DNA. Indeed, it is well established that reduced, labelled misonidazole binds to G residues in DNA but that the binding is several orders of magnitude lower than the observed damage [38]. As mentioned previously, binding is maximal to poly(d[GC]) and absent with poly(d[AT]), the precise opposite of the base specificity for damage [43, 55]. In 1980 Olive clearly showed that the redox potentials of nitroimidazoles correlated positively with their mutagenicity [24], suggesting, at least theoretically, that adduct formation and mutagenicity were correlated. In contrast, the *in vitro* structure–activity data described previously [38, 56] suggest that maximal DNA damage is produced by low potential nitroimidazoles which are (relatively) non-mutagenic. It is to answer the question: is DNA damage produced by reduced nitroimidazoles due to adduct or non-adduct formation that much effort has been directed towards the identification of the reduced intermediate and reductive metabolism which will be discussed elsewhere in some detail (Goldman, this Symposium).

The evidence that DNA damage is produced by a short-lived reduction intermediate of nitroimidazoles comes from many studies using a range of techniques. In general, DNA damage occurs only when reduction is carried out in the presence of DNA and not when DNA is added a few seconds later [60]. Subsequent studies have confirmed this using different techniques and a range of nitroimidazoles [52, 53, 59]. Significantly, chemical reduction of labelled misonidazole and DNA produces the same amount of binding to DNA irrespective of whether DNA is added after or during reduction [61]. These results strongly suggest that DNA damage and macromolecular binding is produced by two different mechanisms; the former by a non-adduct mechanism (possibly involving free radicals) and the latter by adduct formation.

WHAT IS THE REDUCTION INTERMEDIATE RESPONSIBLE FOR DNA DAMAGE?

Reduction of nitroimidazoles is a complex process. Theoretically, the nitro group may accept up to six electrons in a complete reduction to the corresponding amine

$$\mathrm{R{-}NO_2 \xrightarrow{2e} R{-}NO \xrightarrow{2e} R{-}NHOH \xrightarrow{2e} R{-}NH_2} \qquad (1)$$

The detailed process is complex involving the sequential addition of an electron, protonation and disproportionation thus:

$$\mathrm{R{-}NO_2 \longrightarrow R{-}N\dot{O}_2^-} \qquad (2)$$

$$\mathrm{R{-}N\dot{O}_2^- + H^+ \longrightarrow R{-}NO_2\dot{H}} \qquad (3)$$

$$\mathrm{2R{-}NO_2\dot{H} \longrightarrow R{-}NO_2 + R{-}N(OH)_2} \qquad (4)$$

$$\mathrm{R{-}N(OH)_2 \longrightarrow R{-}NO + H_2O} \qquad (5)$$

$$\mathrm{R{-}NO \longrightarrow R{-}N\dot{O}^-} \qquad (6)$$

$$\mathrm{R{-}N\dot{O}^- + H^+ \longrightarrow R{-}NO\dot{H}} \qquad (7)$$

$$\mathrm{R{-}NO\dot{H} + R{-}NO_2\dot{H} \longrightarrow R{-}NHOH + R{-}NO_2} \qquad (8)$$

$$\mathrm{R{-}NHOH \longrightarrow R{-}NHO\dot{H}^-} \qquad (9)$$

$$\mathrm{R{-}NHO\dot{H}^- + 2H^+ \longrightarrow R{-}NH_2 + H_2O} \qquad (10)$$

Despite considerable study by several groups no stable intermediate has been characterized from 5-nitroimidazoles. Metronidazole yields seven reduction products when reduced by xanthine oxidase [62], and these account for less than half the original drug. Electrolytic reduction yields six products, only four of which match those from enzyme studies [40]. No final reduction product produces any cytotoxicity or DNA damage as might be expected and reduction does not proceed to the amine even though the amine derivative of metronidazole has been synthesized and found to be inactive [63]. The amine derivative of misonidazole, however, is produced in hypoxic lung fibroblasts [54], in hypoxic liver cells [64] by radiolytic reduction [48] and by intestinal microflora [53, 54], but whether it is produced by reduction of the hydroxylamine (equations 9 and 10) or by disproportionation of two hydroxylamine derivatives (equation 11) is not known.

$$\mathrm{2R{-}NHOH \longrightarrow R{-}NH_2 + R{-}NO + H_2O} \qquad (11)$$

The azo, azoxy and hydrazo derivatives of misonidazole have also been detected in radiolytic and chemical reduction systems [44, 48, 65]. More recently it has been shown that electrolytic or radiolytic reduction of misonidazole yields the hydroxylamine derivative which is capable of rearrangement to yield glyoxal [47, 52]. This feature of misonidazole may explain the formation of a glyoxal derivative of guanosine [46] and a recently discovered glutathione adduct [45] which could be relevant to the ability of these drugs to deplete cellular non-protein thiols [25]. The rearrangement of the hydroxylamine in an analogous manner to a Bamberger rearrangement has recently been elucidated by McClelland and coworkers [66], but such a feature cannot occur with 5-nitroimidazoles which do not produce glyoxal, nor is glyoxal itself capable of reacting with DNA to form adducts thus suggesting an activated chemically reactive derivative as being the important chemical species in this respect.

One approach adopted to elucidate the short-lived intermediate response for DNA damage is the technique of coulometry which measures the statistical average number of electrons used in the reduction process [40, 67–69]. In general, nitrophenyl drugs such as chloramphenicol give a 6-electron stoichiometry indicating formation to the corresponding amine [70, 71]. The 2-nitroimidazole group of drugs generally give a 4-electron stoichiometry indicating reduction to the hydroxylamine level [41, 67, 69, 72]. In contrast, 5-nitroimidazole drugs give non-integral electron values, typically between 3 and 4 [67, 68, 72]. The exception is satranidazole which behaves as a 2-nitroimidazole in this respect.* One explanation for the non-integral electron stoichiometry of 5-nitroimidazoles is their ability to produce nitrite which is not seen to any extent in 2-nitroimidazoles, nitrofurans or nitrophenyl drugs. Nitrite arises from the decomposition of the nitro radical anion [70, 73]

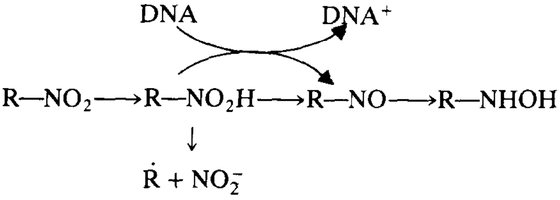
$$\text{R—N}\dot{\text{O}}_2^{-} \longrightarrow \dot{\text{R}} + \text{NO}_2^{-} \qquad (12)$$

A significant feature of the coulometric approach is that if experiments to determine electron stoichiometry are carried out in the presence of the target, i.e. DNA, the electron values decrease and the decrease is related to the % A + T content of the DNA [67–69]. This effect is most clearly seen with 2-nitroimidazoles and nitrophenyl compounds which produce little or no nitrite but is complicated in 5-nitroimidazoles which produce up to 30% nitrite [69, 73, 74]. The inference is that a reduced short-lived intermediate is able to abstract electrons (and protons) from DNA thus accounting for the decreased electron values and the oxidation process occurs in the region of thymidine residues explaining the observed strand breakage and release of thymidine.

Recent work using the ϕX174 transformation assay system† has shown that DNA damage induced by reduced nitroimidazoles is not repaired by the excision repair pathway in *Escherichia coli* which would be expected if adducts were responsible for damage. Moreover, nitroimidazoles in general have little effect on the SOS repair pathway as measured by sister chromatid exchange [75, 76]. This suggests that DNA damage is caused by a mechanism which is not caused by the binding of reduced drug to form stable adducts. Using the same technique a significant finding is that the amount of DNA damage produced by electrolytically reduced nitroimidazoles depends upon the reduction rate, the slower the rate the greater the damage.* The one-electron nitro radical anion of 5-nitroimidazoles decays with second order kinetics whereas the decay of 2-nitroimidazoles is predominantly first order [77]. With 5-nitroimidazoles high reduction rates would tend to favour disproportionation to the parent drug and the nitroso derivative (equations 3 to 5) summarized below

$$2\text{R—N}\dot{\text{O}}_2^{-} \longrightarrow \text{R—NO}_2 + \text{R—NO} + \text{H}_2\text{O} \qquad (13)$$

A similar reaction might also occur for 2-nitroimidazoles preceded, however, by a first order protonation [77]. If the agent responsible for DNA damage was the nitroso or hydroxylamine (or metabolites thereof) their formation would be enhanced at high reduction rates but this is clearly not the case suggesting the one-electron nitro radical anion as a more plausible candidate than nitroso or hydroxylamine derivatives. The model depicted below is speculative



but envisages $\text{R—NO}_2\text{H}$ acting as an electron acceptor (or R—NO_2^{-} as an electron and proton acceptor) from DNA. The model predicts the decreased electron stoichiometry in the presence of the target presumably in the region of thymidine residues in a manner analogous to radiosensitizer-induced DNA damage [78] and also predicts that damage to DNAs of high % A + T would decrease the electron stoichiometry, that damage does not involve adduct formation and therefore cannot be repaired and also predicts decreased damage at high reduction rates due to disproportionation (equation 13).

The relationship between DNA damage produced by electrolytically reduced nitroimidazoles and their reduction potential is a negative one as described earlier [38, 56, 79]. This relationship has a slope coefficient of $9.3 \pm 0.5\ \text{V}^{-1}$ which value is similar to that found for strand breakage and thymine release of $11\ \text{V}^{-1}$ [56]. This correlation may reflect either the relative activity of the protonated radical or its relative stability rather than the rate of reduction as is found for correlations of radiosensitization and hypoxic cytotoxicity [57, 58]. The anomaly that structure–activity correlations of nitroimidazoles in anaerobic or anoxic conditions yields a negative relationship with reduction potential whereas those carried out under hypoxia show a positive correlation is discussed in the following section.

REDUCTION OF NITROIMIDAZOLES IN HYPOXIA

It is well established that the cytotoxicity of a wide range of nitro heterocyclic drugs increases with increased electron affinity in a hypoxic environment. In respect of the action of 5-nitroimidazoles it is now generally accepted that *T. vaginalis* does not exist in a totally anaerobic (anoxic) environment and many resistant *T. vaginalis* show oxygen tolerance which is evident only in aerobic assays [80–82]. Under conditions of hypoxia therefore the influence of oxygen in the environment assumes significance even at relatively low levels because of its high electron affinity and ability to scavenge electrons in the "futile cycle" [83, 84]. In this reaction oxygen accepts an electron from the one-electron nitro radical anion reforming the parent drug and producing superoxide as shown below:

$$\text{R—N}\dot{\text{O}}_2^{-} + \text{O}_2 \longrightarrow \text{R—NO}_2 + \dot{\text{O}}_2^{-} \qquad (14)$$

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* Zahoor, Knight and Edwards, unpublished.

† Lafleur *et al.*, *Int. J. radiat. Biol.* in press; Zahoor *et al.*, submitted to *Int. J. radiat. Oncol. Biol. Phys.*

The "futile cycle" will be determined by the concentration of O_2 in the environment and the relative stability or reactivity of the nitro radical anion to O_2. The 5-nitroimidazole drug metronidazole is intracellularly reduced in *T. vaginalis*. In metronidazole resistant strains Lloyd and Pedersen [85] have recently shown that the nitro radical anion is scavenged (oxidized) more efficiently by O_2 than in sensitive strains thus permitting futile cycling to occur and such a process may limit drug uptake which is dependent upon the rate of intracellular reduction [32]. Rauth and co-workers have also shown that the oxidation of the metronidazole nitro radical anion occurs at a lower O_2 concentration than that required for misonidazole [49]. These observations enable the prediction to be made that those 5-nitroimidazoles of higher reduction potential than metronidazole may be more resistant to futile cycling and thus more effective antitrichomonal agents. This certainly appears to be the case with satranidazole [7].

It is the relative stability of the nitro radical anion to futile cycling in the presence of O_2 which may explain the anomaly of structure–activity correlations in anoxia or anaerobiosis and hypoxia. Thus, in a hypoxic (or partially anaerobic/microaerophilic) environment those nitroimidazoles of low redox potential (5-nitroimidazoles in the main) will have the inherent capacity to engender more DNA damage than those of higher potential but upon reduction the nitro radical anion will be scavenged more efficiently by O_2 thus reducing their effective concentration and limiting drug uptake to the cell. In such a case the structure–activity correlations will show a positive slope because not only are drugs of high reduction potential less sensitive to futile cycling but the overall damage will be solely dependent upon the rate of drug reduction. In contrast, under anoxic or anaerobic conditions futile cycling will not occur and the correlations will be negative reflecting the relative stability or activity of the nitro radical anion.

Much work remains to be done, however, to elucidate further the reactive intermediates and particularly to distinguish between biological effects mediated via adduct and non-adduct mechanisms.

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