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PUSHING THE FRONTIERS OF RADIOBIOLOGY: A SPECIAL FEATURE IN MEMORY OF SIR OLIVER SCOTT AND PROFESSOR JACK FOWLER: REVIEW ARTICLE

Nitroimidazoles as hypoxic cell radiosensitizers and hypoxia probes: misonidazole, myths and mistakes

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ABSTRACT

Nitroimidazoles have been extensively explored as hypoxic cell radiosensitizers but have had limited clinical success, with efficacy restricted by toxicity. However, they have proven clinically useful as probes for tumour hypoxia. Both applications, and probably much of the dose-limiting toxicities, reflect the dominant chemical property of electron affinity or ease of reduction, associated with the nitro substituent in an aromatic structure. This single dominant property affords unusual, indeed extraordinary flexibility in drug or probe design, suggesting further development is possible in spite of earlier limitations, in particular building on the benefit of hindsight and an appreciation of errors made in earlier studies. The most notable errors were: the delay in viewing cellular thiol depletion as a likely common artefact in testing *in vitro*; slow recognition of pH-driven concentration gradients when compounds were weak acids and bases; and a failure to explore the possible involvement of pH and ascorbate in influencing hypoxia probe binding. The experience points to the need to involve a wider range of expertise than that historically involved in many laboratories when studying the effects of chemicals on radiation response or using diagnostic probes.

INTRODUCTION

One of the most highly cited papers in radiobiology was published in this Journal in 1953, when 'Hal' Gray, Oliver Scott and their colleagues at Mount Vernon Hospital drew attention to the possibility of tumour hypoxia as a common cause of failure in radiotherapy.¹ After the obvious approach to overcome this problem was explored—hyperbaric oxygen treatment—chemical substitutes for oxygen in this context were sought. Since oxygen was well known to be highly reactive towards many free radicals, and the latter species are by definition produced by ionizing radiation, chemicals reactive towards free radicals were one logical avenue for exploration. The free, hydrated electron was being extensively studied as an intermediate in water radiolysis by 1960 and the development of methods to follow its reactions directly, available at Mount Vernon by 1962,² doubtless stimulated 'Ged' Adams and his colleagues there to consider substances reactive towards the electron as candidate radiosensitizers.³ One early model of action was the chemical prevention by electron-affinic compounds of recombination of the electron and its counter-ion, thus facilitating damage associated with what might be termed the positive 'hole' or electron-loss species.

The breakthrough leading directly, and quickly, to chemical radiosensitizers selective towards hypoxic cells with potential clinical usefulness came with the identification of 4- (or *para*-) nitroacetophenone as a radiosensitizer.^{4,5} This is a simple chemical structure [Figure 1, (1)] consisting of an aromatic moiety—a benzene ring—substituted with both a very strongly electron-withdrawing group, the nitro function, as well as a weaker but amplifying electron-withdrawing substituent, the acetyl or $-C(=O)CH_3$ group. Nitro compounds based on other aromatic ring systems were already used in medicine and are still widely used today as antibiotics and antiparasitic agents: common examples include nitrofurans such as nitrofurantoin (Figure 1) (2) and nifurtimox (3), and nitroimidazoles such as metro-nidazole (4) and benznidazole (5). Both these nitroheteroaromatic families were soon identified as potential radiosensitizers.^{6,7} Importantly, activity could be modulated by additional aromatic ring substituents: efficacy of nitrobenzenes was correlated with a well-established chemical parameter, the Hammett sigma value, which *quantitatively* reflects the additional electron-attracting power the substituent bestows on the nitroaromatic molecule.⁸

Figure 1. Structures of some of the compounds discussed: (1) 4-nitroacetophenone; (2) nitrofurantoin; (3) nifurtimox; (4) metronidazole; (5) benznidazole; (6) the Mannich base of 4-nitroacetophenone, 'NDPP'; (7) misonidazole (Ro 07-0582); (8) desmethyl misonidazole (Ro 05-9963); (9) etanidazole (SR 2508); (10) pimonidazole, (Ro 03-8799); (11) nimorazole; (12) sanazole (AK-2123); (13) KU-2285; (14) doranidazole (PR-350); (15) 5-chloro-1-methyl-4-nitroimidazole; (16) 1-methyl-2-nitroimidazole-5-carboxaldehyde (L-8711); (17) a 2-nitrobenzimidazole (Ro 21-7982); (18) 1-methyl-2-nitrosoimidazole; (19) nitrosobenzene.

Additionally, the second substituent was a useful 'handle' on which to 'bolt-on' modifications such as a water-solubilizing function with a fairly small change in the overall electron-affinity, facilitating the demonstration of efficacy *in vivo* with the 'Mannich base' analogue of 4-nitroacetophenone ('NDPP'; Figure 1) (6), cf. (1).^{9,10}

The generality of radiosensitizing activity for most nitroaromatic molecules and relative insensitivity towards additional (at

least 'electro-neutral') substituents was underlined by the identification of several 2-nitroimidazoles as radiosensitizers more efficient than the 5-nitroimidazole analogues such as metronidazole (4);¹¹ one such compound, from Roche Products Ltd., 'Ro 07-0582' or misonidazole (Figure 1) (7) was very widely investigated. The 'electron affinity' relationship established by Raleigh et al⁸ was then extended to include a range of nitroaromatic structures when direct measurements of the ease of reduction to the radical-anion (reduction potential)¹² became available.¹³⁻¹⁵

The nitroaromatic structure is susceptible to metabolic changes involving the nitro group accepting electrons from reducing enzymes (flavoprotein nitroreductases), and nitroreduction generally occurs stepwise via an obligate free radical reactive towards oxygen,^{16,17} a blocking or inhibition of reduction by oxygen via these free-radical reactions which occur in around 10 ms in normoxic tissue, a timescale readily calculable from direct kinetic observations.¹⁸ Because the nitro free radical would otherwise lead to formation of a nitrosoaromatic compound which reacts with protein (and non-protein) thiol groups,^{19–21} probably in a few tens of milliseconds,²² nitroreductases lead to the phenomenon of oxygen-sensitive binding via N–S bonds. This is the basis for the widespread use of nitroimidazoles to identify areas of reduced oxygen tension in tissue.^{23–30}

In this brief review, no attempt will be made to summarize all the work on chemical mechanisms of radiosensitization or the related field of the use of the same molecules as chemical probes for tissue hypoxia. Some mechanistic aspects of radiosensitization by nitroaromatic compounds and the chemistry of nitroaromatic hypoxia probes have been discussed elsewhere by the present author.^{22,31–33}

Summaries are available of extensive clinical experience with metronidazole; misonidazole; its more hydrophilic analogues [the desmethyl analogue Ro 05–9963 (Figure 1) (8) and etanidazole, (SR 2508, 9)]; pimonidazole (10); and the 5-nitroimidazole, nimorazole (11).^{34–41} In short, trials with misonidazole, etanidazole and pimonidazole failed to show benefit, while nimorazole was demonstrated to be clearly beneficial in large-scale trials in Denmark. In an overview in 1996,³⁸ quoting a meta-analysis by Overgaard, it was observed that hypoxic cell sensitizers gave an improvement in local control of 3.9% ($p = 0.004$), and ‘The statistical significance is high but many would not regard a margin of 4.6% [this figure included data from hyperbaric oxygen trials] as sufficient to justify the widespread introduction of a complex and/or toxic method of treatment. The greatest importance of the overview is that it confirms that hypoxia can be a cause of radiation failure and demonstrates that benefit can be gained by the use of methods to sensitize hypoxic tumour cells.’ Danish experience with nimorazole and radiotherapy of supraglottic larynx and pharynx carcinoma indicated ‘the nimorazole group showed a significantly better loco-regional control rate than the placebo group (49 vs 33%, $p = 0.002$).’⁴⁰ Smaller clinical trials have been conducted with other nitroarene radiosensitizers, including the 3-nitrotriazole, sanazole (AK-2123, Figure 1) (12); a compound with a fluorinated sidechain (KU-2285, 13); and doranidazole (PR-350, 14).^{42–45} Results were generally disappointing except for sanazole.⁴³

Overall, the administered dose in clinical trials was too small to achieve the high dose-modification factors seen in *in vitro* models, being limited by morbidities including the central and peripheral neuropathy which was well known before 1970 to be a feature of nitroheterocyclic compounds used in medicine and which is still a topic of interest today.⁴⁶ Note that neuropathy was not dose-limiting with either pimonidazole or nimorazole.^{35,40} Other factors which limited the clinical benefits have been debated.⁴⁷

There is general agreement that a method to stratify patients, selecting those with tumours having demonstrable regions of hypoxia, would be of great value. The 2-nitroimidazole moiety has been shown to be selectively retained in hypoxic cells in tissue via a mechanism discussed further below, retention of a molecular fragment providing a measure of hypoxia.^{23–30} Variants of this approach have been exploited widely, not always appreciating factors such as pH and ascorbate, which might influence measurement with all 2-nitroimidazole probes, as discussed below.

It is the intention here only to discuss some aspects which, with hindsight, ought to have been better appreciated at the time but which should still be instructive today. Three distinct problem areas are addressed. It should be noted that while examples are drawn from experience with nitroimidazoles, most of the points discussed have wider relevance.

PROBLEM 1: THIOLS

Depletion of cellular thiols by *some* nitroimidazoles (and probably by *most* when hypoxic cells are exposed to high concentrations of them for prolonged periods)

Thiols are well known to be radioprotective molecules, so depletion of this protective pool is a potential source of uncertainty. If restricted to the target hypoxic cells and hypoxia is only present in tumour cells, well and good; but if thiol levels in normal tissues are reduced, then obviously a potential problem arises. However, routine testing of the reactivity of nitroimidazole and related radiosensitizers towards thiols was seldom carried out. At the Gray Laboratory the problem was strikingly apparent when a 4-nitroimidazole of measured low electron affinity was found to radiosensitize much more efficiently than expected.⁴⁸ The molecule had an additional 5-chloro substituent (Figure 1) (15), and since chemists recognize this as a ‘leaving group’, a possible (but completely wrong) explanation based on the properties of the radical-anion, similar to the behaviour of nitrobenzyl halide radical-anions, was initially suggested.⁴⁹ The explanation was much simpler: cellular thiols were displacing the halide ‘leaving group’ (in a reaction catalysed by glutathione-S-transferases); the behaviour was also exhibited by other 4-nitroimidazoles with substituents such as $-\text{SO}_2\text{CH}_3$.^{50–54} Some nitro compounds, including 1-methyl-2-nitroimidazole substituted with 5-CHO (Figure 1) (16), a 2-nitrobenzimidazole (17), and the 5-nitrofuran nifurtimox (3) react with thiols with the *nitro* substituent being displaced as nitrite.^{53,55} Other, broadly similar examples of radiosensitization by thiol-reactive nitroimidazoles were reported in different laboratories, for brevity not further discussed here.^{52,56,57} Unsurprisingly, depletion of intracellular glutathione significantly enhances the efficacy of nitroimidazole radiosensitizers,⁵⁸ since a basic tenet is that these radiosensitizers are oxygen-mimetic and a model for the oxygen effect is kinetic competition between oxygen and thiols for a free radical.

Thiols and ‘simple arithmetic’

If the nitro substituent is displaced by thiol (an electron-donating function), why do such nitro compounds still radiosensitize—at least in the usual test systems *in vitro*? The answer lies in simple arithmetic, and shows why the numerous studies

of thiol-reactive nitro compounds really amounted to little more than a wild goose chase—or rather, chases. As illustrated previously,^{32,59} comparing the amount (moles) of thiols in cells tested in a petri dish with the amount (moles) of thiol-reactive chemical added shows that a huge excess of thiol-reactive nitro compound is present compared to the thiol pool, so that the latter is completely depleted in contrast to the test chemical, the concentration of which has hardly changed. The reverse scenario is expected at tissue cell densities and practical drug doses *in vivo*. In the author's view, this behaviour is illustrative of a general problem in testing the effects of chemicals at low cell densities, and the failure to draw up a 'balance sheet' assessing both moles and molarity of chemical added and target affected may be the source of other false leads in drug research. It is salutary to note that very much reduced radiosensitization by 4-nitroacetophenone was observed at high cell densities *in vitro* (compared to cell suspensions),⁶⁰ long before the present author realized the importance of cell density effects in routine testing: 'simple arithmetic'.⁵⁹

The implications of thiol reactivity

To be fair, it *was* recognized at an early date that some nitro compounds could deplete 'hydrogen donor' compounds in cells.⁶¹ The effects of thiols on radiosensitization by metronidazole were reported in 1974.⁶² Thiol depletion was one of the unusual characteristics of 'NDPP' (Figure 1) (6) *in vitro*.⁶³ Hall et al described the enhanced radiosensitization after prolonged exposure of cells to misonidazole, termed it the 'Extra Enhancement Effect' and linked it to thiol depletion.⁶⁴ The use of *E. coli* strains with different nitroreductase activity further demonstrated a clear link between the enhanced radiosensitization and nitroreduction, and confirmation of the involvement of thiols.^{65,66} Cellular thiol measurements were a feature in comparing misonidazole with a hydrophilic analogue with a nucleoside substituent.⁶⁷

However, there was a general failure both to assess such effects as a matter of routine for all compounds in the testing procedure and (especially) to view them as artefacts of *in vitro* testing, which would not be translatable *in vivo* because of cell density effects. In consequence, some conclusions previously thought to be very well established should now be viewed with at least some measure of caution. Regrettably, this includes the highly cited 'electron affinity relationship'.^{14,15} While the basic relationship is undoubtedly sound, *at least* one or two of the compounds tested at the higher end of the reduction potential scale are now known to be directly thiol-reactive on the timescale of the test studies; but *all* have the potential to reduce the cellular thiol pool during the pre-irradiation exposure while the cells were being deoxygenated.

The timescale of testing (particularly the pre-irradiation anoxic exposure to nitro compound) is clearly important here, and in an ideal world avoidance of secondary effects by short pre-irradiation incubation would have avoided these artefacts. In practice, ensuring the test system is thoroughly de-oxygenated takes time and a modified testing procedure would be required. Importantly, even where direct thiol reactivity of the nitro compound is very slow (and it certainly is with misonidazole),⁵¹

as soon as the test cells become hypoxic, cellular nitroreductases 'kick in', and the resulting metabolism produces, via the radical-anion, the nitroso 2-electron reduction product which is highly reactive towards thiols. Synthesis of 1-methyl-2-nitrosoimidazole (Figure 1) (18) (and painstaking identification of 'downstream' reduction products and their properties) enabled the properties of the thiol-depleting intermediate(s) to be studied in detail.^{20,68–70} The nitrosoimidazole reduction product was proposed to be the intermediate responsible for the hypoxic cytotoxicity of 2-nitroimidazoles.⁷¹ Rapid loss of the nitroso compound was observed on adding either glutathione or ascorbate.²⁰ A non-toxic concentration of 25 μ M of the nitrosoimidazole (18) depleted GSH in cells *in vitro* to 10–20% of control levels within 5 min.⁶⁹

Whether some thiol depletion via hypoxic metabolism involving nitroreductases (aside from direct reactions of some compounds with thiols) was a factor influencing the data defining the 'electron affinity' relationship is not known, but it seems quite likely. The more electron-affinic compounds are reduced faster by reduced flavins or flavoproteins.^{72–74} Since anaerobic toxicity is very likely associated with nitroreduction, it is unsurprising that a redox relationship was found for anaerobic cytotoxicity.⁷⁵

Critically, careful time-resolved experiments involving irradiation after only a short pre-irradiation exposure to misonidazole revealed that 'although the rate of development of misonidazole sensitization [as a function of exposure time] is high, the maximum enhancement ratio achieved up to 0.5 s is not greater than ~1.7, even for concentrations which on incubation for many minutes could exhibit enhancements of nearly the maximum observed for oxygen, 2.8'.⁷⁶ Other rapid-mix studies showed broadly similar effects.^{77,78} Earlier work involving pre-irradiation exposure of 3–4 min had also demonstrated significantly lower radiosensitization by misonidazole than found after longer anoxic incubation.⁷⁹ Hence the reports of very high radiation dose-modifying factors achievable by nitroimidazoles at non-cytotoxic concentrations very probably led to an over-expectation of their potential, the response likely including effects from hypoxic metabolism, thiol depletion, and the cell density or 'simple arithmetic' problem outlined above.

A reviewer has pointed out that temperature is also an important variable when assessing radiosensitizers. Almost all measurements of radiosensitization by nitroimidazoles and similar compounds involved irradiation at room temperature following exposing the cells (with radiosensitizer) to nitrogen or nitrogen/carbon dioxide for perhaps an hour or more. Enzyme-catalysed reactions may well have a different temperature dependence compared to the free-radical reactions involved in radiosensitization. An instructive example of the value of comparing effects at different temperatures is the similar behaviour of misonidazole and an analogue with an alkylating function on the sidechain when assessed at 4°C, but not at room temperature, when the alkylating compound showed markedly enhanced effects.⁸⁰ The reviewer also drew attention to the lack of further radiosensitization by nitroarenes in cells irradiated under aerobic conditions, in contrast to the increase in aerobic radiosensitivity afforded by

thiol reagents such as diethylmaleate. While this might suggest significant thiol depletion is not a major artefact in most studies, without measurement of cellular thiols, comparison is difficult.

It is possible that the slope of the well-known 'electron affinity' plot for radiosensitization would be lower than reported if thiol-depleting effects were excluded. This is important because in evaluating simple chemical models for radiosensitization (the effects of electron affinity on the rates constants for reactions involving addition to model carbon-centred radicals), redox relationships [log (rate constant) vs reduction potential] have been compared. These showed slopes considerably lower ($\sim 3\text{--}4\text{ V}^{-1}$) than found for radiosensitization efficiency ($\sim 8\text{ V}^{-1}$).^{81,82} Lower slopes of the 'electron affinity' plots for radiosensitization, ~ 6 or 7 V^{-1} , were found if concentrations to achieve enhancement ratios of 1.2 or 1.4, rather than 1.6 were analysed,^{14,15} analysis of the smaller effects possibly closer to the 'true' redox relationship because of fewer artefacts from thiol depletion.

PROBLEM 2: PH

Effects of weak acid or base functionality of compounds on extra/intracellular and subcellular concentration gradients

Statistical analysis of the radiosensitizing efficiencies *in vitro* of 35 nitroaromatic compounds failed to reveal a significant dependence on the lipophilicity of the molecules,¹⁴ and decreasing the lipophilicity below that of misonidazole to reduce neuronal exposure was evaluated as a strategy to improve the therapeutic ratio.^{83,84} However, as mentioned above, a weakly basic substituent had been utilized earlier to enhance the aqueous solubility of 4-nitroacetophenone ('NDPP', Figure 1, 6). While this showed enhanced activity *in vitro* compared to the parent structure (1), deviating from the relationship between radiosensitization and the Hammett indicator of electron affinity,⁸ this unusual activity was not translated to *in vivo* models, and a 'disparity between the enhancement ratios obtained from the *in vivo* and *in vitro* studies' was noted,⁸⁵ also found in another laboratory.⁸⁶ More detailed studies of 'NDPP' (6) showed thiol depletion and enhanced intracellular uptake of 'NDPP' *in vitro*, presumably (since radiolabel was measured rather than the specific compounds) reflecting both thiol reactivity and label retention, as well as pH-induced gradients (see below).⁶³ Surprisingly, thiols displaced the basic moiety ($-\text{N}(\text{CH}_3)_2$), evidently activated towards nucleophilic displacement by the strongly electron-withdrawing properties of the rest of the molecule.

Later, analogues of the 2-nitroimidazole, misonidazole, terminated with weakly basic functional groups were explored, with some compounds showing up to an order of magnitude increase in radiosensitizing efficiency *in vitro* over that expected from the reduction potential. *Aerobic* cytotoxicity was used to help identify promising compounds, from which pimonidazole, a misonidazole analogue with a basic substituent (*N*-piperidino, $\text{pK}_a \sim 8.7$, Figure 1) (10) was selected for further study.^{87,88} Presumably displacement of the basic function by thiols, as found with 'NDPP' (6), does not occur with pimonidazole because of the slightly lower basicity and the somewhat weaker electron-withdrawing properties, overall, of the rest of the molecule.

Pimonidazole was shown to exhibit rather variable radiosensitization compared to misonidazole in mouse models, with much less dramatic differences than seen *in vitro*.^{89,90} Promising tumour concentrations of pimonidazole were reported in patients,⁹¹ with high concentrations relative to normal tissue.⁹² However, a clinical trial with pimonidazole (0.75 g m^{-2} , about 19 mg kg^{-1} with each of typically 25 treatments in a normal fractionation schedule) in the radiotherapy of carcinoma of the cervix showed no benefit.³⁵ It is still not clear why human trials were disappointing, but one property that might lead to (at the least) significant differences between cell, mouse and man is the pH in different tissue compartments and indeed in different sub-cellular organelles. Pimonidazole reduced blood flow in an experimental tumour model by 30–40% at a single dose of 500 mg kg^{-1} (about 25-fold higher in mass terms than that used with each treatment in the clinical trial).⁹³ Direct comparison of dosing between mouse and man in mass terms is inappropriate, and a scaling factor of about 12 has been suggested,⁹⁴ so 500 mg kg^{-1} in the mouse might be equivalent to about 40 mg kg^{-1} in humans; however, the clinical trial report described the pimonidazole dose used in the animal study as 'very similar (on a dose per surface area basis) to that given patients in this trial'.³⁵

Despite pH-induced concentration gradients of weak acids and bases across a lipid membrane being well known in pharmacology, it was not until years after the first *in vitro* experiments with nitroimidazoles with weak acid/base functionality that measurement of *average* intracellular concentrations showed that for the same medium concentrations, pimonidazole was concentrated approximately 3-fold intracellularly compared to misonidazole in the standard *in vitro* test system, an effect which steadily disappeared if the medium pH was reduced slightly.⁹⁵ Other misonidazole analogues with weakly basic moieties on the sidechain concentrated intracellularly *in vitro*, the effect varying with extracellular pH.^{80,96}

The *average* intracellular drug concentration may not accurately reflect the concentration at the target site if, e.g. the drug concentrates in acidic sub-cellular organelles. By modifying the intralysosomal pH, it was possible to reduce the measured average intracellular concentration of pimonidazole by a factor of two, although this had little effect upon sensitization.⁹⁷ Methods exist, using both fluorescent probes and direct measurements, to 'report' effective concentrations of radiosensitizers in the immediate vicinity of DNA.^{98,99} The studies suggested that pimonidazole did indeed concentrate near DNA relative to misonidazole. Whether the model systems are representative of the pH gradients in human tumours is not known.

An instructive study with misonidazole analogues having alkylating functionality after rearrangement of a basic substituent showed the potential of exploiting pH gradients in targeting melanotic melanoma.¹⁰⁰ Comparison of uptake into melanotic cell lines *in vitro* of a neutral nitroimidazole and pimonidazole showed a disparity between uptake of the latter and its radiosensitization effect, being dependent on cell line and cell cycle.¹⁰¹ A detailed study of labelled pimonidazole uptake *in vivo* in a melanotic tumour model showed that after 24 h, picnotic areas

of tumours had levels of label around 40 times greater than background, coincident with areas of highest apparent melanin content. However, the authors pointed out that as these cells were probably non-clonogenic, pimonidazole was not recommended for use in melanoma radiotherapy.¹⁰²

Clearly, pH is an important variable in influencing drug distribution at both macro and microscopic levels, but the effects are complex and require detailed study. As an aside, time-dependent, pH-driven concentration gradients also drive the intracellular accumulation *in vitro* of polyamine thiol radioprotectors such as WR-1065, the dephosphorylated active form of amifostine.¹⁰³ Hence enhanced uptake *in vitro*, which might not be reflected in the same way *in vivo* because of 'simple arithmetic', can lead to misinterpretation of the relative efficacy of different thiols as radioprotectors from *in vitro* screening—leaving aside different effects from thiol/disulphide exchange *in vitro* and *in vivo*,^{103–105} again because of 'simple arithmetic'.

Nitroimidazole hypoxia 'stains' may be influenced by differences in pH as well as pO_2

It is widely accepted that the basis for the use of nitroimidazoles to reflect tissue hypoxia is competition between two reaction pathways: (i) the protective reaction with oxygen of the nitro radical-anion (the obligate initial product of nitroreduction by many flavoproteins), which reflects the lower electron affinity of the nitro compound compared to oxygen, and (ii) the 'natural' reaction pathway(s) of the nitro radical which occur in the absence of oxygen. Apart from reactivity towards oxygen, nitro radicals are rather unreactive except for the self-reaction or disproportionation to form a nitrosoarene. The latter compounds react rapidly with thiol functions to retain a fragment of the probe intracellularly,²¹ detectable by antibody staining or radiolabel.^{23–30} However, the 'natural' lifetimes of nitro radicals are a function of pH: a pH reduction of 0.3 results in a factor of two decrease in lifetime.¹⁰⁶ Exactly the same pH-dependent behaviour is exhibited by oxygen radical-anions (superoxide),¹⁰⁷ except that the self-reaction is catalysed by superoxide dismutase, whereas nitro radicals are not substrates for this enzyme.^{108,109} The shorter lifetime of nitro radicals in regions of lower pH dictates that more oxygen is required to inhibit staining or retention via the nitroreduction pathway, other things being equal (ignoring, *e.g.* any possible pH dependence of nitroreductases enzyme activity). Hence hypoxia probes may, in part, also be reflecting pH gradients.

Incidentally, the 'futile cycling' of oxygen that occurs in the first of the two competing reactions does, of course, consume oxygen—hardly an ideal characteristic of an oxygen monitor—so the more sensitive the method for detecting the bound residue, the better. Enhancement of oxygen utilization by misonidazole was reported many years ago,¹¹⁰ but seems to have been quietly forgotten. Further, the method assumes constant nitroreductases activity and cofactor levels across the sample,²⁸ as well as 'time-averaging' hypoxia as noted below.

Another confounding factor might be the slower reaction of nitrosoarene with thiols at lower pH, since the reactive form of

the thiol is the thiolate anion, and the pK_a for dissociation of thiol to thiolate is generally in the region of ~9 so that (again) a reduction in pH of 0.3 below physiological would slow down the binding reaction by a factor of two. If there are competing targets for the nitrosoarene—and ascorbate may be one—then the rate of the binding reaction might be important. However, even very fast reactions of thiols can be catalysed by glutathione-S-transferases; an example is the reaction with glutathione of the toxic quinoneimine metabolite of paracetamol (acetaminophen).¹¹¹ If such catalysis is a feature with nitrosoimidazoles the binding reaction might not be as pH-sensitive as the uncatalysed reaction where only thiolate is effectively reacting. These issues have been discussed briefly some years ago by the present author in this Journal, including illustration of the possible involvement of other reduction intermediates such a hydroxylaminoimidazoles.²²

Clearly, there are questions over the details of the mechanism of oxygen-sensitive binding of nitroimidazoles which remain generally overlooked. As with the oxygen effect in radiobiology,¹¹² time is also an important parameter in the use of hypoxia 'stains'. Aside from the obvious point that the accumulation of the bound residue that is assumed to reflect hypoxia 'time averages' oxygen levels so that regions labelled as hypoxic may be reoxygenated even at the time of sampling, the timescale of reactions of two critical precursors of the bound 'stain' are fundamental to the method.

The first important timescale is the lifetime of the nitro radical-anion, which as a general rule for nitroarene radicals is inversely proportional to their steady-state concentrations, resulting in the 'protecting' electron transfer to oxygen occurring at very low levels of oxygen indeed, far lower than that of the bulk of radioresistant hypoxic cells. However, radical-anions of 2- but not 5-nitroimidazoles, or nitrobenzenes, exhibit unusual properties, with lifetimes which are largely independent of their concentrations.^{106,109,113} The numerical values characterizing essentially exponential radical decay and the rate constant for reaction with oxygen make 2-nitroimidazoles particularly well suited both as oxygen 'reporters' and as hypoxia-activated triggers for bioreductive drugs,²² a point returned to below. Entirely consistent with the chemical-kinetic parameters, it was found in a model reducing system that several micromolar oxygen was required to inhibit the reduction of 2-nitroimidazoles, in contrast to around one-tenth micromolar oxygen being sufficient to inhibit reduction of metronidazole or 4-nitroacetophenone.¹¹⁴ In agreement with the chemical model, some protection against the hypoxic cytotoxicity of metronidazole towards cells *in vitro* was afforded by as little as 0.01% v/v (about one-tenth micromolar) oxygen, whereas over 10-fold higher levels of oxygen were needed to inhibit toxicity in comparable experiments with a much more electron-affinic 5-nitrofurans.¹¹⁵ The radical-anions of nitrofurans reacts with oxygen more than an order of magnitude slower than those of metronidazole or misonidazole.^{18,109,113}

The second timescale to consider when evaluating potential hypoxia 'stains' is that of the reaction forming the bound

product. If this is too long, then the reactive intermediate, probably the nitrosoimidazole with 2-nitroimidazole probes, could diffuse out of the cell and be trapped in cells distant from the site of production and potentially not hypoxic at all. Fortunately, it can be predicted that the reaction 'sphere' for nitrosoarene trapping might be around 10–20 μM , of the order of a single cell diameter. The chemical kinetics of the reaction enables a likely timescale of trapping to be deduced, and translated into approximate distance using the Einstein-Smoluchowski equation, which relates the root-mean-square diffusion distance of a species to time, depending only on an easily estimated diffusion coefficient.²²

PROBLEM 3: ASCORBATE

Ascorbate is present in human tissue but generally not in cultured cells

Ascorbate is present in some human tissues at levels of at least several hundred micromolar (in brain, far higher, as described below), but it is normally virtually undetectable in cultured cells *in vitro*. Even if added to cell cultures, transport (uptake) is active rather than passive, slow, and temperature-dependent.¹¹⁶ Ascorbate has been shown to partially counteract the radiosensitizing effect of misonidazole in thiol-depleted human fibrosarcoma cells,¹¹⁷ and in a murine fibrosarcoma.¹¹⁸ Whether standard *in vitro* test systems (where the thiol pool is normal) omitted an important antioxidant, an omission which *might* have resulted in an exaggeration of the radiosensitizing effect of added nitroimidazoles is debatable, but the possibility should have been considered more widely.

As noted above, nitrosoarenes, key intermediates in hypoxia-sensitive binding of nitroimidazoles and related compounds, are highly reactive towards ascorbate as well as thiols.^{19,20,119} Whether ascorbate can protect against label binding following reaction with thiol moieties in the use of nitroimidazoles as hypoxia probes depends on the relative values of the rate constants multiplied by the reactant concentrations for the two competing reactions. Of course, if ascorbate is absent, there is no competition. This means it *may* be unwise to 'calibrate' the oxygen response with such probes using cultured cells, expecting the results to be mirrored *in vivo*. Directly relevant chemical studies are lacking, but fairly good guidance as to the possible competition between thiols and ascorbate for reaction with nitrosoarenes can be found from literature data on the chemical kinetics of reaction of the representative simpler model, nitrosobenzene (Figure 1) (19), with glutathione and ascorbate.^{19,119} The former reaction appears to involve an intermediate adduct,¹⁹ but taking the reported rate constant for the formation of this at 37°C, pH 7.4 and comparing this with the rate constant for reaction of ascorbate with nitrosobenzene at 37°C suggests the two antioxidants have very similar reactivity towards nitrosoarenes at physiological pH. Since the total thiol pool in tissue usually, but not always, is at a considerably higher concentration than that of ascorbate (see below), the chemical-kinetic data thus suggest a relatively minor role of ascorbate in intercepting nitroreduction intermediates at ascorbate levels typical of skeletal muscle, for example. It should be stressed that chemical-kinetic data for

reaction of thiols and ascorbate with nitrosoimidazoles are not available and thus the potential involvement of ascorbate in influencing probe binding using nitroimidazoles remains somewhat uncertain.

Is ascorbate protective or causative in the neurotoxicity of nitroimidazoles?

Since the dose-limiting neurotoxic characteristics of some nitroimidazoles are also a feature of other nitroaromatics, and the cytotoxicity of such compounds is much higher in hypoxia than in oxic cells, it is reasonable to assume that toxicity is associated with reduction of the nitro group. Biochemical markers for the dose-limiting neurotoxicity of nitroarenes have been discussed in a number of studies.^{120–123} The enzymes β -glucuronidase and β -galactosidase in rat nervous tissue are elevated after administration of either metronidazole, misonidazole or nitrofurantoin, an effect also observed with the neurotoxin methylmercury and consistent with peripheral nerve damage. Vitamin E or thiamine pyrophosphate afforded some protection.¹²³ If the protective role of vitamin E involves formation of the vitamin phenoxyl radical, it is important to note that the vitamin is not depleted because of rapid recycling of the radical by ascorbate.¹²⁴ There is direct chemical interaction between nitroarenes and catecholamine neurotransmitters (forming nitro radical-anions and hence superoxide radicals), a mechanistic route meriting further exploration.¹²⁵ Alternative neuroprotectors which might be worth investigating in the context of nitroimidazoles include hydroxytyrosol, a dietary phenol found in olive oil.¹²⁶

A logical extension of enquiring whether ascorbate has any effect in oxygen-sensitive, nitroreductase-associated binding of nitro compounds is to consider whether co-administration of ascorbate could permit somewhat higher doses of nitroimidazoles to be administered to humans, and if it could, would there be an effect on radiosensitization efficiency, as suggested from studies in a mouse model.¹¹⁸ Of course, the (unproven) assumption is made that dose-limiting human toxicity is associated with insufficient protection of nitroreduction by physiological oxygen levels. If, on the other hand, it is elevation of oxidative stress via 'futile cycling' of oxygen driven by nitroreductases that is the cause of neurotoxicity (as suggested for cytotoxicity *in vitro*),¹²⁷ then high-dose ascorbate is hardly likely to improve matters.

Ascorbate levels in human tissues vary widely.¹²⁸ In cerebrospinal fluid or skeletal muscle it is around 200 μM or higher,^{128–130} but compartmentalization under homeostatic control results in levels in neurons and glia which are very much higher than this, around 10 and 1 mM, respectively.¹²⁹ The average ascorbate content in adult cerebral cortex is linearly dependent on neuron density, being around four times higher in mouse than human.¹²⁹ While knowledge of the role of ascorbate in brain chemistry is considerable,¹³⁰ its potential role in the peripheral neurotoxicity that is the characteristic dose-limiting feature of *some* nitroarene radiosensitizers remains unproven, and chemical intervention of the nitrosoarene reduction intermediate is at best speculative; in any case, homeostatic control may defeat attempts to elevate ascorbate in the target tissue.

Nitroarenes are potential pro-oxidants in catalysing superoxide-associated oxidative stress in neurons via reduction by ascorbate. Studies of the effects of ascorbate on nitroarene cytotoxicity *in vitro* might suggest enhancing ascorbate concentrations to reduce neurotoxicity to be an unfruitful avenue to pursue: it was shown that ascorbate enhanced the hypoxic cytotoxicity of misonidazole,^{131–133} consistent with generation of nitro radical-anions from nitroarenes by ascorbate in anaerobic chemical systems, although only seen directly at very high pH with nitroimidazoles.¹³⁴ The reaction of ascorbate with nitroarenes to generate the ascorbate radical and the nitro radical-anion is thermodynamically very unfavourable, since the reduction potentials of the nitroarenes are around 0.7 V lower than that of the ascorbate radical; removal of the radical products from the reaction drives the unfavourable reaction. Even so, reduction of misonidazole by ascorbate is very slow at pH 7 and 25°C in anoxia,¹³⁵ but it would have been much more informative to have measured superoxide formation at 37°C in the presence of (say) 80 µM oxygen. Whether these experiments are at all relevant to likely effects in aerobic tissue *in vivo* is unclear: in fact, administration of ascorbic acid glucoside (but not ascorbic acid) *did* reduce neurotoxicity of the 3-nitrotriazole, sanazole (Figure 1) (12) in a mouse model.¹³⁶

Interestingly, the 5-nitrofuran nifurtimox, widely used in humans against Chagas' disease (American trypanosomiasis), is a substrate for aldehyde dehydrogenase 2; inhibitors of this enzyme protect against toxicity in a zebrafish model,¹³⁷ with significant implications for the use of nitrofurans in man since low-cost inhibitors of this enzyme are available.¹³⁸ Nifurtimox is currently under Phase 2 clinical trial in chemotherapy of neuroblastoma.¹³⁹ Is it coincidence that neural tissues have high levels of ascorbate? As expected, enhancement of neural tumour cell killing by nifurtimox using the glutathione synthetase inhibitor, buthionine sulfoxamine, has been demonstrated.¹⁴⁰ The claim 'we have made a new discovery that nifurtimox ... can function as a hypoxia-activated cytotoxin' in a paper published in 2017 may be technically correct, but it hardly comes as a surprise.¹⁴¹ While there is certainly a sense of *déjà vu* in reading these reports (and the lesson of 'simple arithmetic' has apparently not been learned by some of the researchers), these more recent studies of nitrofuran-associated toxicity, and ongoing concern over neurotoxicity in the treatment of Chagas' disease,¹⁴² may have wider implications in the present context.

High dose ascorbate is currently being investigated as a source of hydrogen peroxide for therapeutic purposes, plasma concentrations of several millimolar being achievable after intravenous administration of ~10 g ascorbate in humans.^{128,143,144} The safety of high dose ascorbate is quite well established, and while the focus of the recent work is to enhance oxidative stress for therapeutic purposes, the potential reduction in tumour hypoxia which might result from hydrogen peroxide delivery (and subsequent breakdown to oxygen) via ascorbate should not be overlooked. Recent work has shown ascorbate (4 g kg⁻¹) to act as a radiosensitizer in a mouse model of pancreatic cancer,¹⁴⁵ which should be compared with much earlier work with ascorbate involving mouse tumour models and normal tissues.¹¹⁸ A

comparison of endogenous ascorbate levels in common mouse and human tumours is needed.

Much of the above discussion on the potential roles of ascorbate in the use of nitroimidazole radiosensitizers or hypoxia probes stems in part from a comparison of just two reported rate constants characterizing reactivity of nitrosobenzene with thiols and ascorbate.^{19,119} This points not only to the need for more chemical-kinetic, biochemical and toxicological studies to follow up on the early leads of Biaglow, Koch, McClelland, Rauth, Suit and others who set the scene for studying the roles of ascorbate. The real question to be asked is why the radiobiological community generally appeared to think it did not matter in studying radiobiological effects, or in assessing hypoxia using chemical probes, that the most well-known antioxidant is generally completely absent in *in vitro* models, and is not at all well characterized in *in vivo* models.

CONCLUSIONS

Of course, this paper should really be subtitled 'with the benefit of hindsight' or 'being wise after the event'. The author was himself involved in making some of the 'mistakes' referred to in the title. But are other scientists now making the same mistakes, even if sophisticated new methods are available and newer avenues for therapeutic gain are being explored? For example, the critical kinetic parameters required for successful hypoxia probes discussed above, notably the concentration-independent 'natural' lifetimes characteristic of 2-nitroimidazole radicals but not of many other nitroarene radicals, suggests focus should be on this specific nitroaromatic system not only for hypoxia stains, but also as the basis for reductively activated 'triggers' for delivering therapeutic molecules via radical fragmentation (at least, if the same target populations for both assessing hypoxia and releasing cytotoxins are desired). Some other nitroaromatic cores may target only a small, almost anoxic populations of cells, a specificity also critically depending on the rates of radical fragmentation. Protocols recommended for assessing candidate bioreductive drugs failed to include methods to characterize such key chemical-kinetic parameters even although kinetic factors are absolutely central to rational drug design.¹⁴⁶

Fully 40 years on after 'NDPP' and misonidazole, radiosensitization by new analogues are being described without reporting concomitant assessment of the effects (if any) of the compounds on the cellular thiol pool under the testing conditions, or measurements of direct reactivity (if any) of the compounds with glutathione and possible acceleration by glutathione transferases.¹⁴⁷ In contrast, 40 years on a much higher degree of sophistication is now exhibited when discussing reductases and the hypoxic microenvironment in the context of hypoxia probes and hypoxia-activated prodrugs.^{28,148}

The issues discussed here underline how important it is to draw on a wide area of expertise in drug development. Some groups involved in the search for clinically useful radiosensitizers benefited from the tradition established by Hal Gray and Oliver Scott at Mount Vernon and encouraged by Jack Fowler, and included a range of skills in the same building. Others drew on nearby expertise, illustrated by the impressive work in Toronto. Collaboration

with Roche Products Ltd. was a key factor in moving on from nitroacetophenone,¹¹ and a partnership involving another commercial company afforded early insight into the biochemistry of nitroimidazole neurotoxicity.^{120–123} However, even with multi-disciplinary and inter-laboratory collaborations progress was slow and errors were made. It would be a pity if lessons were not learned from these mistakes. By 1980, what was needed to progress hypoxic cell radiosensitizers was not more look-alike drugs nor more radiobiology, but more biochemical pharmacology and more detailed studies of the mechanisms, especially of the chemical-kinetic parameters that ultimately control efficacy and toxicity. Ironically, research on oxygen-dependent radiosensitivity has moved towards molecular biology,¹⁴⁹ a direction unlikely to further chemical modification of free-radical damage as the solution to the problem of hypoxia in radiotherapy.

Nitroarene radiosensitizers are fail-safe drugs with a well-defined target, high specificity, and offering quite extraordinary flexibility in drug design. It should not be beyond the capability of science to identify compounds which have appropriate free-radical reactivity but which are either resistant to unwanted metabolic activation or amenable to pharmacological suppression of toxicity.

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REFERENCES

- Gray LH, Conger AD, Ebert M, Hornsey S, Scott OCA. The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy. *Br J Radiol* 1953; **26**: 638–638. doi: <https://doi.org/10.1259/0007-1285-26-312-638>
- Hart EJ, Boag JW. Absorption spectrum of the hydrated electron in water and in aqueous solutions. *J Am Chem Soc* 1962; **84**: 4090–5. doi: <https://doi.org/10.1021/ja00880a025>
- Adams GE, Cooke MS. Electron-affinic sensitization. I. A structural basis for chemical radiosensitizers in bacteria. *Int J Radiat Biol Relat Stud Phys Chem Med* 1969; **15**: 457–71. doi: <https://doi.org/10.1080/09553006914550741>
- Adams GE, Asquith JC, Dewey DL, Foster JL, Michael BD, Willson RL. Part II: para-Nitroacetophenone: a radiosensitizer for anoxic bacterial and mammalian cells. *Int J Radiat Biol Relat Stud Phys Chem Med* 1971; **19**: 575–85. doi: <https://doi.org/10.1080/09553007114550751>
- Chapman JD, Webb RG, Borsa J. Radiosensitization of mammalian cells by p-nitroacetophenone. I. Characterization in asynchronous and synchronous populations. *Int J Radiat Biol Relat Stud Phys Chem Med* 1971; **19**: 561–73. doi: <https://doi.org/10.1080/09553007114550741>
- Chapman JD, Reuvers AP, Borsa J, Petkau A, McCalla DR. Nitrofurans as radiosensitizers of hypoxic mammalian cells. *Cancer Res* 1972; **32**: 2616–24.
- Foster JL, Willson RL. Radiosensitization of anoxic cells by metronidazole. *Br J Radiol* 1973; **46**: 234–5. doi: <https://doi.org/10.1259/0007-1285-46-543-234>
- Raleigh JA, Chapman JD, Borsa J, Kremers W, Reuvers AP. Radiosensitization of mammalian cells by p-nitroacetophenone. III. Effectiveness of nitrobenzene analogues. *Int J Radiat Biol Relat Stud Phys Chem Med* 1973; **23**: 377–87. doi: <https://doi.org/10.1080/09553007314550431>
- Adams GE, Asquith JC, Watts ME, Smithen CE. Radiosensitization of hypoxic cells in vitro: a water-soluble derivative of pararnitroacetophenone. *Nat New Biol* 1972; **239**: 23–4. doi: <https://doi.org/10.1038/newbio239023a0>
- Denekamp J, Michael BD. Preferential sensitization of hypoxic cells to radiation in vivo. *Nat New Biol* 1972; **239**: 21–3. doi: <https://doi.org/10.1038/newbio239021a0>
- Asquith JC, Watts ME, Patel K, Smithen CE, Adams GE. Electron affinic sensitization. V. Radiosensitization of hypoxic bacteria and mammalian cells *in vitro* by some nitroimidazoles and nitropyrazoles. *Radiat Res* 1974; **60**: 108–18. doi: <https://doi.org/10.2307/3574010>
- Wardman P. Reduction potentials of one-electron couples involving free radicals in aqueous solution. *J Phys Chem Ref Data* 1989; **18**: 1637–755. doi: <https://doi.org/10.1063/1.555843>
- Wardman P, Clarke ED. One-electron reduction potentials of substituted nitroimidazoles measured by pulse radiolysis. *J Chem Soc, Faraday Trans 1* 1976; **72**: 1377–90. doi: <https://doi.org/10.1039/f19767201377>
- Adams GE, Flockhart IR, Smithen CE, Stratford IJ, Wardman P, Watts ME. Electron-affinic sensitization. VII. A correlation between structures, one-electron reduction potentials, and efficiencies of nitroimidazoles as hypoxic cell radiosensitizers. *Radiat Res* 1976; **67**: 9–20. doi: <https://doi.org/10.2307/3574491>
- Adams GE, Clarke ED, Flockhart IR, Jacobs RS, Sehmi DS, Stratford IJ, et al. Structure-activity relationships in the development of hypoxic cell radiosensitizers. I. Sensitization efficiency. *Int J Radiat Biol Relat Stud Phys Chem Med* 1979; **35**: 133–50. doi: <https://doi.org/10.1080/09553007914550151>
- Mason RP, Holtzman JL. The role of catalytic superoxide formation in the O₂ inhibition of nitroreductase. *Biochem Biophys Res Commun* 1975; **67**: 1267–74. doi: [https://doi.org/10.1016/0006-291X\(75\)90163-1](https://doi.org/10.1016/0006-291X(75)90163-1)
- Mason RP, Holtzman JL. The mechanism of microsomal and mitochondrial nitroreductase. Electron spin resonance evidence for nitroaromatic free radical intermediates. *Biochemistry* 1975; **14**: 1626–32. doi: <https://doi.org/10.1021/bi00679a013>
- Wardman P, Clarke ED. Oxygen inhibition of nitroreductase: electron transfer from nitro radical-anions to oxygen. *Biochem Biophys Res Commun* 1976; **69**: 942–9. doi: [https://doi.org/10.1016/0006-291X\(76\)90464-2](https://doi.org/10.1016/0006-291X(76)90464-2)
- Eyer P. Reactions of nitrosobenzene with reduced glutathione. *Chem Biol Interact* 1979; **24**: 227–39. doi: [https://doi.org/10.1016/0009-2797\(79\)90011-5](https://doi.org/10.1016/0009-2797(79)90011-5)
- Noss MB, Panicucci R, McClelland RA, Rauth AM. Preparation, toxicity and mutagenicity of 1-methyl-2-nitrosoimidazole. A toxic 2-nitroimidazole reduction product. *Biochem Pharmacol* 1988; **37**: 2585–93. doi: [https://doi.org/10.1016/0006-2952\(88\)90250-X](https://doi.org/10.1016/0006-2952(88)90250-X)

21. Raleigh JA, Koch CJ. Importance of thiols in the reductive binding of 2-nitroimidazoles to macromolecules. *Biochem Pharmacol* 1990; **40**: 2457–64. doi: [https://doi.org/10.1016/0006-2952\(90\)90086-Z](https://doi.org/10.1016/0006-2952(90)90086-Z)
22. Wardman P. The importance of radiation chemistry to radiation and free radical biology (The 2008 Silvanus Thompson Memorial Lecture. *Br J Radiol* 2009; **82**: 89–104. doi: <https://doi.org/10.1259/bjr/60186130>
23. Chapman JD, Franko AJ, Sharpin J. A marker for hypoxic cells in tumours with potential clinical applicability. *Br J Cancer* 1981; **43**: 546–50. doi: <https://doi.org/10.1038/bjc.1981.79>
24. Koch CJ, Evans SM, Lord EM. Oxygen dependence of cellular uptake of EF5 [2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl)acetamide] : analysis of drug adducts by fluorescent antibodies vs bound radioactivity. *Br J Cancer* 1995; **72**: 869–74. doi: <https://doi.org/10.1038/bjc.1995.426>
25. Raleigh JA, Dewhirst MW, Thrall DE. Measuring tumor hypoxia. *Semin Radiat Oncol* 1996; **6**: 37–45. doi: [https://doi.org/10.1016/S1053-4296\(96\)80034-8](https://doi.org/10.1016/S1053-4296(96)80034-8)
26. Raleigh JA, Chou SC, Arteel GE, Horsman MR. Comparisons among pimonidazole binding, oxygen electrode measurements, and radiation response in C3H mouse tumors. *Radiat Res* 1999; **151**: 580–9. doi: <https://doi.org/10.2307/3580034>
27. Busk M, Jakobsen S, Horsman MR, Mortensen LS, Iversen AB, Overgaard J, et al. PET imaging of tumor hypoxia using ¹⁸F-labeled pimonidazole. *Acta Oncol* 2013; **52**: 1300–7. doi: <https://doi.org/10.3109/0284186X.2013.815797>
28. Koch CJ, Evans SM. Optimizing hypoxia detection and treatment strategies. *Semin Nucl Med* 2015; **45**: 163–76. doi: <https://doi.org/10.1053/j.semnuclmed.2014.10.004>
29. Kumar P, Bacchu V, Wiebe LI. The chemistry and radiochemistry of hypoxia-specific, radiohalogenated nitroaromatic imaging probes. *Semin Nucl Med* 2015; **45**: 122–35. doi: <https://doi.org/10.1053/j.semnuclmed.2014.10.005>
30. Fleming IN, Manavaki R, Blower PJ, West C, Williams KJ, Harris AL, et al. Imaging tumour hypoxia with positron emission tomography. *Br J Cancer* 2015; **112**: 238–50. doi: <https://doi.org/10.1038/bjc.2014.610>
31. Wardman P. The mechanism of radiosensitization by electron-affinic compounds. *Radiat Phys Chem* 1987; **30**(5–6): 423–32. doi: [https://doi.org/10.1016/1359-0197\(87\)90111-1](https://doi.org/10.1016/1359-0197(87)90111-1)
32. Wardman P. Chemical radiosensitizers for use in radiotherapy. *Clin Oncol* 2007; **19**: 397–417. doi: <https://doi.org/10.1016/j.clon.2007.03.010>
33. Wardman P. Electronic-affinic radiosensitizers. In: Greenberg MM, ed. *Radical and radical ion reactivity in nucleic acid chemistry*. New York: John Wiley & Sons; 2009. pp. 295–324.
34. Dische S. A review of hypoxic cell radiosensitization. *Int J Radiat Oncol Biol Phys* 1991; **20**: 147–52. doi: [https://doi.org/10.1016/0360-3016\(91\)90151-S](https://doi.org/10.1016/0360-3016(91)90151-S)
35. A trial of Ro 03-8799 (pimonidazole) in carcinoma of the uterine cervix: an interim report from the Medical Research Council Working Party on advanced carcinoma of the cervix. *Radiother Oncol* 1993; **26**: 93–103. doi: [https://doi.org/10.1016/0167-8140\(93\)90089-Q](https://doi.org/10.1016/0167-8140(93)90089-Q)
36. Overgaard J. Clinical evaluation of nitroimidazoles as modifiers of hypoxia in solid tumors. *Oncol Res* 1994; **6**: 509–18.
37. Lee DJ, Cosmatos D, Marcial VA, Fu KK, Rotman M, Cooper JS, et al. Results of an RTOG phase III trial (RTOG 85-27) comparing radiotherapy plus etanidazole with radiotherapy alone for locally advanced head and neck carcinomas. *Int J Radiat Oncol Biol Phys* 1995; **32**: 567–76. doi: [https://doi.org/10.1016/0360-3016\(95\)00150-W](https://doi.org/10.1016/0360-3016(95)00150-W)
38. Saunders M, Dische S. Clinical results of hypoxic cell radiosensitisation from hyperbaric oxygen to accelerated radiotherapy, carbogen and nicotinamide. *Br J Cancer* 1996; **74**(Suppl XXVII): S271–8.
39. Eschwege F, Sancho-Garnier H, Chassagne D, Brisgand D, Guerra M, Malaise EP, et al. Results of a European randomized trial of etanidazole combined with radiotherapy in head and neck carcinomas. *Int J Radiat Oncol Biol Phys* 1997; **39**: 275–81. doi: [https://doi.org/10.1016/S0360-3016\(97\)00327-1](https://doi.org/10.1016/S0360-3016(97)00327-1)
40. Overgaard J, Hansen HS, Overgaard M, Bastholt L, Berthelsen A, Specht L, et al. A randomized double-blind phase III study of nimorazole as a hypoxic radiosensitizer of primary radiotherapy in supraglottic larynx and pharynx carcinoma. Results of the Danish head and neck cancer study (DAHANCA) protocol 5-85. *Radiother Oncol* 1998; **46**: 135–46. doi: [https://doi.org/10.1016/S0167-8140\(97\)00220-X](https://doi.org/10.1016/S0167-8140(97)00220-X)
41. Overgaard J. Hypoxic radiosensitization: adored and ignored. *J Clin Oncol* 2007; **25**: 4066–74. doi: <https://doi.org/10.1200/JCO.2007.12.7878>
42. Shibamoto Y, Sugie C, Ito M, Ogino H. The Japanese experiences with hypoxia-targeting pharmacoradiotherapy: from hypoxic cell sensitizers to radiation-activated prodrugs. *Expert Opin Pharmacother* 2004; **5**: 2459–67. doi: <https://doi.org/10.1517/14656566.5.12.2459>
43. Dobrowsky W, Huigol NG, Jayatilake RS, Kizilbash NI, Okkan S, Kagiya VT, et al. AK-2123 (Sanazol) as a radiation sensitizer in the treatment of stage III cervical cancer: results of an IAEA multicentre randomised trial. *Radiother Oncol* 2007; **82**: 24–9. doi: <https://doi.org/10.1016/j.radonc.2006.11.007>
44. Nishimura Y, Nakagawa K, Takeda K, Tanaka M, Segawa Y, Tsujino K, et al. Phase I/II trial of sequential chemoradiotherapy using a novel hypoxic cell radiosensitizer, doranidazole (PR-350), in patients with locally advanced non-small-cell lung cancer (WJTOG-0002). *Int J Radiat Oncol Biol Phys* 2007; **69**: 786–92. doi: <https://doi.org/10.1016/j.ijrobp.2007.04.008>
45. Horsman MR, Overgaard J. The impact of hypoxia and its modification of the outcome of radiotherapy. *J Radiat Res* 2016; **57**(Suppl 1): i90–i98. doi: <https://doi.org/10.1093/jrr/rrw007>
46. Goolsby TA, Jakeman B, Gaynes RP. Clinical relevance of metronidazole and peripheral neuropathy: a systematic review of the literature. *Int J Antimicrob Agents* 2017; [Epub ahead of print]. doi: <https://doi.org/10.1016/j.ijantimicag.2017.08.033>
47. Brown JM. Hypoxic cell radiosensitizers: the end of an era? Regarding Lee et al., IJROBP 32:567-576; 1995. *Int J Radiat Oncol Biol Phys* 1995; **32**: 883–5. doi: [https://doi.org/10.1016/0360-3016\(95\)00186-3](https://doi.org/10.1016/0360-3016(95)00186-3)
48. Watts ME, Jacobs RS. Some examples of anomalous radiosensitizing behaviour of electron-affinic compounds in vitro. *Br J Cancer Suppl* 1978; **3**: 80–3.
49. Clarke ED, Wardman P. Are ortho-substituted 4-nitroimidazoles a new generation of radiation-induced arylating agents? *Int J Radiat Biol Relat Stud Phys Chem Med* 1980; **37**: 463–8. doi: <https://doi.org/10.1080/09553008014550561>
50. Adams GE, Fielden EM, Hardy C, Millar BC, Stratford IJ, Williamson C. Radiosensitization of hypoxic mammalian cells in vitro by some 5-substituted-4-nitroimidazoles. *Int J Radiat Biol Relat Stud Phys Chem Med* 1981; **40**: 153–61. doi: <https://doi.org/10.1080/09553008114551031>
51. Wardman P. The kinetics of the reaction of 'anomalous' 4-nitroimidazole radiosensitizers with thiols. *Int J Radiat Biol Relat Stud Phys Chem Med* 1982;

- 41: 231–5. doi: <https://doi.org/10.1080/09553008214550261>
52. Stratford IJ, Adams GE, Hardy C, Hoe S, O'Neill P, Sheldon PW. Thiol reactive nitroimidazoles: radiosensitization studies in vitro and in vivo. *Int J Radiat Biol Relat Stud Phys Chem Med* 1984; **46**: 731–45. doi: <https://doi.org/10.1080/09553008414551971>
 53. Watts ME, Dennis MF, Stratford MR, Wardman P. Glutathione-reactive nitro compounds as radiosensitizers: mechanistic and therapeutic implications. *Int J Radiat Oncol Biol Phys* 1986; **12**: 1135–8. doi: [https://doi.org/10.1016/0360-3016\(86\)90243-9](https://doi.org/10.1016/0360-3016(86)90243-9)
 54. Wardman P. Thiol reactivity towards drugs and radicals: some implications in the radiotherapy and chemotherapy of cancer. In: Chatgililoglu C, Asmus KD, eds. *Sulfur-centered reactive intermediates in chemistry and biology*. New York: Plenum Press; 1990. pp. 415–27.
 55. Díaz EG, Montalto de Mecca M, Castro JA. Reactions of nifurtimox with critical sulfhydryl-containing biomolecules: their potential toxicological relevance. *J Appl Toxicol* 2004; **24**: 189–95. doi: <https://doi.org/10.1002/jat.970>
 56. Astor M, Hall EJ, Biaglow JE, Parham JC. Newly synthesized hypoxia-mediated drugs as radiosensitizers and cytotoxic agents. *Int J Radiat Oncol Biol Phys* 1982; **8**: 75–83. doi: [https://doi.org/10.1016/0360-3016\(82\)90388-1](https://doi.org/10.1016/0360-3016(82)90388-1)
 57. Stratford IJ, Hoe S, Adams GE, Hardy C, Williamson C. Abnormal radiosensitizing and cytotoxic properties of ortho-substituted nitroimidazoles. *Int J Radiat Biol Relat Stud Phys Chem Med* 1983; **43**: 31–43. doi: <https://doi.org/10.1080/09553008314550031>
 58. Hodgkiss RJ, Middleton RW. Enhancement of misonidazole radiosensitization by an inhibitor of glutathione biosynthesis. *Int J Radiat Biol Relat Stud Phys Chem Med* 1983; **43**: 179–83. doi: <https://doi.org/10.1080/09553008314550201>
 59. Wardman P, Clarke ED. Redox properties and rate constants in free-radical mediated damage. *Br J Cancer Suppl* 1987; **8**(Suppl VIII): 172–7.
 60. Agnew DA, Skarsgard LD. Chemical radiosensitization of anoxic mammalian cells: effect of cell concentration. *Radiat Res* 1974; **57**: 246–59. doi: <https://doi.org/10.2307/3573831>
 61. Chapman JD, Reuvers AP, Borsa J, Greenstock CL. Chemical radioprotection and radiosensitization of mammalian cells growing in vitro. *Radiat Res* 1973; **56**: 291–306. doi: <https://doi.org/10.2307/3573667>
 62. Asquith JC, Foster JL, Willson RL, Ings R, McFadzean JA. Metronidazole ("Flagyl"). A radiosensitizer of hypoxic cells. *Br J Radiol* 1974; **47**: 474–81. doi: <https://doi.org/10.1259/0007-1285-47-560-474>
 63. Wong TW, Whitmore GF. A comparison of radiation-sensitizing ability and cell uptake for NDPP and Ro-07-0582. *Radiat Res* 1977; **71**: 132–48. doi: <https://doi.org/10.2307/3574718>
 64. Hall EJ, Astor M, Biaglow J, Parham JC. The enhanced sensitivity of mammalian cells to killing by X rays after prolonged exposure to several nitroimidazoles. *Int J Radiat Oncol Biol Phys* 1982; **8**: 447–51. doi: [https://doi.org/10.1016/0360-3016\(82\)90658-7](https://doi.org/10.1016/0360-3016(82)90658-7)
 65. Anderson RF, Patel KB. Metabolic potentiation of the radiosensitization of hypoxic bacterial cells afforded by nitroaromatic compounds. *Radiat Res* 1983; **93**: 516–24. doi: <https://doi.org/10.2307/3576030>
 66. Anderson RF, Patel KB, Stratford MR. The influence of thiols on the pre-irradiation incubation effect of nitroimidazoles in *E. coli* cells. *Int J Radiat Biol Relat Stud Phys Chem Med* 1985; **48**: 485–94. doi: <https://doi.org/10.1080/09553008514551551>
 67. Agrawal KC, Rupp WD, Rockwell S. Radiosensitization, pharmacokinetics, and toxicity of a 2-nitroimidazole nucleoside (RA-263). *Radiat Res* 1986; **105**: 227–39. doi: <https://doi.org/10.2307/3576548>
 68. McClelland RA, Panicucci R, Rauth AM. Products of reductions of 2-nitroimidazoles. *J Am Chem Soc* 1987; **109**: 4308–14. doi: <https://doi.org/10.1021/ja00248a028>
 69. Bérubé LR, Farah S, McClelland RA, Rauth AM. Effect of 1-methyl-2-nitrosoimidazole on intracellular thiols and calcium levels in Chinese hamster ovary cells. *Biochem Pharmacol* 1991; **42**: 2153–61. doi: [https://doi.org/10.1016/0006-2952\(91\)90351-5](https://doi.org/10.1016/0006-2952(91)90351-5)
 70. Bérubé LR, Farah S, McClelland RA, Rauth AM. Depletion of intracellular glutathione by 1-methyl-2-nitrosoimidazole. *Int J Radiat Oncol Biol Phys* 1992; **22**: 817–20. doi: [https://doi.org/10.1016/0360-3016\(92\)90531-L](https://doi.org/10.1016/0360-3016(92)90531-L)
 71. Mulcahy RT, Gipp JJ, Ublacker GA, Panicucci R, McClelland RA. Cytotoxicity and glutathione depletion by 1-methyl-2-nitrosoimidazole in human colon cancer cells. *Biochem Pharmacol* 1989; **38**: 1667–71. doi: [https://doi.org/10.1016/0006-2952\(89\)90315-8](https://doi.org/10.1016/0006-2952(89)90315-8)
 72. Clarke ED, Wardman P, Goulding KH. Anaerobic reduction of nitroimidazoles by reduced flavin mononucleotide and by xanthine oxidase. *Biochem Pharmacol* 1980; **29**: 2684–7. doi: [https://doi.org/10.1016/0006-2952\(80\)90087-8](https://doi.org/10.1016/0006-2952(80)90087-8)
 73. Clarke ED, Goulding KH, Wardman P. Nitroimidazoles as anaerobic electron acceptors for xanthine oxidase. *Biochem Pharmacol* 1982; **31**: 3237–42. doi: [https://doi.org/10.1016/0006-2952\(82\)90556-1](https://doi.org/10.1016/0006-2952(82)90556-1)
 74. Orna MV, Mason RP. Correlation of kinetic parameters of nitroreductase enzymes with redox properties of nitroaromatic compounds. *J Biol Chem* 1989; **264**: 12379–84.
 75. Adams GE, Stratford IJ, Wallace RG, Wardman P, Watts ME. Toxicity of nitro compounds toward hypoxic mammalian cells in vitro: dependence on reduction potential. *J Natl Cancer Inst* 1980; **64**: 555–60. doi: <https://doi.org/10.1093/jnci/64.3.555>
 76. Whillans DW, Hunt JW. A rapid-mixing comparison of the mechanisms of radiosensitization by oxygen and misonidazole in CHO cells. *Radiat Res* 1982; **90**: 126–41. doi: <https://doi.org/10.2307/3575801>
 77. Kandaiya S. Radioprotection of radiation damage fixed by misonidazole in Chinese hamster cells—a rapid-mix study. *Radiat Res* 1986; **105**: 272–5. doi: <https://doi.org/10.2307/3576552>
 78. Kandaiya S, Adams GE, Fielden EM, Stratford IJ. Time-dependent components in radiation sensitization of Chinese hamster cells by misonidazole—a rapid mix study. In: Booz J, Ebert H. G, Hartfield H. D, eds. *Seventh symposium on microdosimetry*. London: Harwood Academic Publishers; 1981. pp. 1117–30.
 79. Ling CC, Michaels HB, Epp ER, Peterson EC. Interaction of misonidazole and oxygen in the radiosensitization of mammalian cells. *Int J Radiat Oncol Biol Phys* 1980; **6**: 583–9. doi: [https://doi.org/10.1016/0360-3016\(80\)90386-7](https://doi.org/10.1016/0360-3016(80)90386-7)
 80. Walling JM, Stratford IJ, Adams GE, Silver AR, Ahmed I, Jenkins TC, et al. Studies on the mechanisms of the radiosensitizing and cytotoxic properties of RSU-1069 and its analogues. *Int J Radiat Oncol Biol Phys* 1986; **12**: 1083–6. doi: [https://doi.org/10.1016/0360-3016\(86\)90231-2](https://doi.org/10.1016/0360-3016(86)90231-2)
 81. Wardman P, Clarke ED. Electron transfer and radical-addition in the radiosensitization and chemotherapy of hypoxic cells. In: Breccia A, Fowler J. F, eds. *New chemo and radiosensitizing drugs*. Bologna: lo Scarabeo; 1985. pp. 21–38.
 82. Sharma GK, Madhava Rao BS, O'Neill P. Redox dependence of the reaction

- of α -alkoxyalkyl radicals with a series of oxidants. *J Phys Chem B* 2009; **113**: 2207: 2207: 11. doi: <https://doi.org/10.1021/jp807994b>
83. Brown JM, Workman P. Partition coefficient as a guide to the development of radiosensitizers which are less toxic than misonidazole. *Radiat Res* 1980; **82**: 171–90. doi: <https://doi.org/10.2307/3575246>
 84. Stratford MR, Minchinton AI, Dische S, Saunders MI, Anderson P. Desmethylmisonidazole (Ro 05-9963): clinical pharmacokinetics after multiple oral administration. *Int J Radiat Oncol Biol Phys* 1982; **8**: 377–9. doi: [https://doi.org/10.1016/0360-3016\(82\)90643-5](https://doi.org/10.1016/0360-3016(82)90643-5)
 85. Sheldon PW, Smith AM. Modest radiosensitization of solid tumours in C3H mice by the hypoxic cell radiosensitizer NDPP. *Br J Cancer* 1975; **31**: 81–8. doi: <https://doi.org/10.1038/bjc.1975.10>
 86. Whitmore GF, Gulyas S, Varghese AJ. Studies on the radiation-sensitizing action of NDPP, a sensitizer of hypoxic cells. *Radiat Res* 1975; **61**: 325–41. doi: <https://doi.org/10.2307/3574050>
 87. Adams GE, Ahmed I, Clarke ED, O'Neill P, Parrick J, Stratford IJ, et al. Structure-activity relationships in the development of hypoxic cell radiosensitizers. III. Effects of basic substituents in nitroimidazole sidechains. *Int J Radiat Biol Relat Stud Phys Chem Med* 1980; **38**: 613: 613: 26. doi: <https://doi.org/10.1080/09553008014551451>
 88. Smithen CE, Clarke ED, Dale JA, Jacobs RS, Wardman P, Watts ME. Novel (nitro-1-imidazolyl) alkanolamines as potential radiosensitizers with improved therapeutic properties. In: Brady L. W, ed. *Radiation sensitizers. Their use in the clinical management of cancer*. New York: Masson; 1980. pp. 22–32.
 89. Williams MV, Denekamp J, Minchinton AI, Stratford MR. In vivo assessment of basic 2-nitroimidazole radiosensitizers. *Br J Cancer* 1982; **46**: 127–37. doi: <https://doi.org/10.1038/bjc.1982.174>
 90. Hill SA, Fowler JE, Minchinton AI, Stratford MR, Denekamp J. Radiosensitization of a mouse tumour by Ro 03-8799: acute and protracted administration. *Int J Radiat Biol Relat Stud Phys Chem Med* 1983; **44**: 143–50. doi: <https://doi.org/10.1080/09553008314550941>
 91. Dische S, Saunders MI, Bennett MH, Dunphy EP, Des Rochers C, Stratford MR, et al. A comparison of the tumour concentrations obtainable with misonidazole and Ro 03-8799. *Br J Radiol* 1986; **59**: 911–7. doi: <https://doi.org/10.1259/0007-1285-59-705-911>
 92. Dische S, Bennett MH, Orchard R, Stratford MR, Wardman P. The uptake of the radiosensitizing compound Ro 03-8799 (pimonidazole) in human tumors. *Int J Radiat Oncol Biol Phys* 1989; **16**: 1089–92. doi: [https://doi.org/10.1016/0360-3016\(89\)90923-1](https://doi.org/10.1016/0360-3016(89)90923-1)
 93. Chaplin DJ, Horsman MR. Tumor blood flow changes induced by chemical modifiers of radiation response. *Int J Radiat Oncol Biol Phys* 1992; **22**: 459–62. doi: [https://doi.org/10.1016/0360-3016\(92\)90853-A](https://doi.org/10.1016/0360-3016(92)90853-A)
 94. Nair AB, Jacob S. A simple practice guide for dose conversion between animals and human. *J Basic Clin Pharm* 2016; **7**: 27–31. doi: <https://doi.org/10.4103/0976-0105.177703>
 95. Dennis MF, Stratford MR, Wardman P, Watts ME. Cellular uptake of misonidazole and analogues with acidic or basic functions. *Int J Radiat Biol Relat Stud Phys Chem Med* 1985; **47**: 629–43. doi: <https://doi.org/10.1080/09553008514550871>
 96. Walling J, Stratford IJ, Adams GE, Stephens MA. Dual-function radiation sensitizers and bioreductive drugs: factors affecting cellular uptake and sensitizing efficiency in analogues of RSU 1069. *Int J Radiat Biol Relat Stud Phys Chem Med* 1988; **53**: 641–9. doi: <https://doi.org/10.1080/09553008814550961>
 97. Stratford MR, Dennis MF, Watts ME, Watfa RR. Radiosensitizer-DNA interactions. *Br J Radiol* 1988; **61**: 860–1. doi: <https://doi.org/10.1259/0007-1285-61-729-860-b>
 98. Wardman P, Dennis MF, White J. A probe for intracellular concentrations of drugs: delayed fluorescence from acridine orange. *Int J Radiat Oncol Biol Phys* 1989; **16**: 935–8. doi: [https://doi.org/10.1016/0360-3016\(89\)90890-0](https://doi.org/10.1016/0360-3016(89)90890-0)
 99. Stratford MR, Dennis MF, Watts ME, Watfa RR, Woodcock M. Radiosensitizer-DNA interactions in relation to intracellular uptake. *Int J Radiat Oncol Biol Phys* 1989; **16**: 1007–10. doi: [https://doi.org/10.1016/0360-3016\(89\)90904-8](https://doi.org/10.1016/0360-3016(89)90904-8)
 100. Walling JM, Deacon J, Holliday S, Stratford IJ. High uptake of RSU 1069 and its analogues into melanotic melanomas. *Cancer Chemother Pharmacol* 1989; **24**: 28–32. doi: <https://doi.org/10.1007/BF00254101>
 101. El Gamoussi R, Stratford IJ, Guichard M. Relationship between intracellular concentration and radiosensitizing effect of pimonidazole and etanidazole on two human melanoma cell lines. *Int J Radiat Biol* 1993; **63**: 27–36. doi: <https://doi.org/10.1080/09553009314550051>
 102. El Gamoussi R, Threadgill MD, Prade M, Stratford IJ, Guichard M. Relationship between the melanin content of a human melanoma cell line and its radiosensitivity and uptake of pimonidazole. *Cancer Chemother Pharmacol* 1993; **31**: 277–82. doi: <https://doi.org/10.1007/BF00685671>
 103. Dennis MF, Stratford MRL, Wardman P, White J. Thiols and antioxidants in radiobiology: chemical and bioanalytical problems. In: Seymour C. B, Mothersill C, eds. *New developments in fundamental and applied radiobiology*. London: Taylor & Francis; 1991. pp. 328–33.
 104. Dennis MF, Stratford MR, Wardman P, Watfa RR. Increase in intracellular cysteine after exposure to dithiothreitol: implications in radiobiology. *Int J Radiat Biol* 1989; **56**: 877–83. doi: <https://doi.org/10.1080/09553008914552351>
 105. Wardman P, Dennis MF, Stratford MR, White J. Extracellular: intracellular and subcellular concentration gradients of thiols. *Int J Radiat Oncol Biol Phys* 1992; **22**: 751–4. doi: [https://doi.org/10.1016/0360-3016\(92\)90517-L](https://doi.org/10.1016/0360-3016(92)90517-L)
 106. Wardman P. Lifetimes of the radical-anions of medically-important nitroaryl compounds in aqueous solution. *Life Chem Rep* 1985; **3**: 22–8.
 107. Bielski BHJ, Cabelli DE, Arudi RL. Reactivity of HO₂/O₂⁻ radicals in aqueous solution. *J Phys Chem Ref Data* 1985; **14**: 1041–100.
 108. Wardman P. Specificity of superoxide dismutase in catalysing redox reactions: a pulse radiolysis study. In: Edwards HE, Navaratnam S, Parsons BJ, Phillips GO, eds. *Radiation biology and chemistry: research developments*. Amsterdam: Elsevier; 1979. pp. 189–96.
 109. Wardman P. Some reactions and properties of nitro radical-anions important in biology and medicine. *Environ Health Perspect* 1985; **64**: 309–20. doi: <https://doi.org/10.1289/ehp.8564309>
 110. Biaglow JE. The effect of hypoxic cell radiosensitizing drugs on cellular oxygen utilization. *Pharmacol Ther* 1980; **10**: 283–99. doi: [https://doi.org/10.1016/0163-7258\(80\)90084-4](https://doi.org/10.1016/0163-7258(80)90084-4)
 111. Coles B, Wilson I, Wardman P, Hinson JA, Nelson SD, Ketterer B. The spontaneous and enzymatic reaction of N-acetyl-p-benzoquinonimine with glutathione: a stopped-flow kinetic study. *Arch Biochem Biophys* 1988; **264**: 253–60. doi: [https://doi.org/10.1016/0003-9861\(88\)90592-9](https://doi.org/10.1016/0003-9861(88)90592-9)

112. Wardman P. Time as a variable in radiation biology: the oxygen effect. *Radiat Res* 2016; **185**: 1–3. doi: <https://doi.org/10.1667/RR14323.1>
113. Wardman P. Chemistry of nitroarene and aromatic N-oxide radicals. In: Alfassi ZB, ed. *The Chemistry of N-Centered Radicals*. New York: John Wiley & Sons; 1998. pp. 615–61.
114. Rauth AM, McClelland RA, Michaels HB, Battistella R. The oxygen dependence of the reduction of nitroimidazoles in a radiolytic model system. *Int J Radiat Oncol Biol Phys* 1984; **10**: 1323–6. doi: [https://doi.org/10.1016/0360-3016\(84\)90341-9](https://doi.org/10.1016/0360-3016(84)90341-9)
115. Mohindra JK, Rauth AM. Increased cell killing by metronidazole and nitrofurazone of hypoxic compared to aerobic mammalian cells. *Cancer Res* 1976; **36**: 930–6.
116. Stratford MR, Hodgkiss RJ. Radioprotection by ascorbate *in vitro*: temperature-dependent uptake. *Br J Radiol* 1986; **59**: 83–4. doi: <https://doi.org/10.1259/0007-1285-59-697-83>
117. Hodgkiss RJ, Stratford MR. Competitive dose-modification between ascorbate and misonidazole in human and hamster cells: effects of glutathione depletion. *Int J Radiat Biol* 1988; **54**: 601–10. doi: <https://doi.org/10.1080/09553008814552041>
118. Okunieff P, Suit HD. Toxicity, radiation sensitivity modification, and combined drug effects of ascorbic acid with misonidazole in vivo on FSaII murine fibrosarcomas. *J Natl Cancer Inst* 1987; **79**: 377–81.
119. Uršić S, Vrčec V, Ljubas D, Vinković I. Interaction of L-ascorbate with substituted nitrosobenzenes. Role of the ascorbate 2-OH group in antioxidant reactions. *New J Chem* 1998; **22**: 221–3. doi: <https://doi.org/10.1039/a709241e>
120. Rose GP, Dewar AJ, Stratford IJ. A biochemical method for assessing the neurotoxic effects of misonidazole in the rat. *Br J Cancer* 1980; **42**: 890–9. doi: <https://doi.org/10.1038/bjc.1980.337>
121. Rose GP, Dewar AJ, Moffett BJ, Stratford IJ. A biochemical assessment of the neurotoxicity of the radiosensitizing drug misonidazole in the rat. *Clin Toxicol* 1981; **18**: 1411–26. doi: <https://doi.org/10.3109/15563658108990350>
122. Rose GP, Dewar AJ, Stratford IJ. A biochemical neurotoxicity study relating the neurotoxic potential of metronidazole and nitrofurantoin with misonidazole. *Int J Radiat Oncol Biol Phys* 1982; **8**: 781–5. doi: [https://doi.org/10.1016/0360-3016\(82\)90734-9](https://doi.org/10.1016/0360-3016(82)90734-9)
123. Rose GP, Dewar AJ, Stratford IJ. Protection against misonidazole-induced neuropathy in rats: a biochemical assessment. *Toxicol Lett* 1983; **17**: 181–5. doi: [https://doi.org/10.1016/0378-4274\(83\)90055-3](https://doi.org/10.1016/0378-4274(83)90055-3)
124. Packer JE, Slater TF, Willson RL. Direct observation of a free radical interaction between vitamin E and vitamin C. *Nature* 1979; **278**: 737–8. doi: <https://doi.org/10.1038/278737a0>
125. Rao DN, Mason RP. Generation of nitro radical anions of some 5-nitrofurans, 2- and 5-nitroimidazoles by norepinephrine, dopamine, and serotonin. A possible mechanism for neurotoxicity caused by nitroheterocyclic drugs. *J Biol Chem* 1987; **262**: 11731–6.
126. Mohan V, Das S, Rao SB. Hydroxytyrosol, a dietary phenolic compound forestalls the toxic effects of methylmercury-induced toxicity in IMR-32 human neuroblastoma cells. *Environ Toxicol* 2016; **31**: 1264–75. doi: <https://doi.org/10.1002/tox.22134>
127. Brezden CB, Horn L, McClelland RA, Rauth AM. Oxidative stress and 1-methyl-2-nitroimidazole cytotoxicity. *Biochem Pharmacol* 1998; **56**: 335–44. doi: [https://doi.org/10.1016/S0006-2952\(98\)00158-0](https://doi.org/10.1016/S0006-2952(98)00158-0)
128. Du J, Cullen JJ, Buettner GR. Ascorbic acid: chemistry, biology and the treatment of cancer. *Biochim Biophys Acta* 2012; **1826**: 443–57. doi: <https://doi.org/10.1016/j.bbcan.2012.06.003>
129. Rice ME. Ascorbate regulation and its neuroprotective role in the brain. *Trends Neurosci* 2000; **23**: 209–16. doi: [https://doi.org/10.1016/S0166-2236\(99\)01543-X](https://doi.org/10.1016/S0166-2236(99)01543-X)
130. Figueroa-Méndez R, Rivas-Arancibia S. Vitamin C in health and disease: its role in the metabolism of cells and redox state in the brain. *Front Physiol* 2015; **6**: 397. doi: <https://doi.org/10.3389/fphys.2015.00397>
131. Joseph PD, Palic B, Skarsgard LD. Ascorbate-enhanced cytotoxicity of misonidazole. *Nature* 1978; **271**: 370–2. doi: <https://doi.org/10.1038/271370a0>
132. Koch CJ, Howell RL, Biaglow JE. Ascorbate anion potentiates cytotoxicity of nitro-aromatic compounds under hypoxic and anoxic conditions. *Br J Cancer* 1979; **39**: 321–9. doi: <https://doi.org/10.1038/bjc.1979.56>
133. Taylor YC, Rauth AM. Sulphydryls, ascorbate and oxygen as modifiers of the toxicity and metabolism of misonidazole *in vitro*. *Br J Cancer* 1980; **41**: 892–900. doi: <https://doi.org/10.1038/bjc.1980.166>
134. Rao DN, Harman L, Motten A, Schreiber J, Mason RP. Generation of radical anions of nitrofurantoin, misonidazole, and metronidazole by ascorbate. *Arch Biochem Biophys* 1987; **255**: 419–27. doi: [https://doi.org/10.1016/0003-9861\(87\)90410-3](https://doi.org/10.1016/0003-9861(87)90410-3)
135. Clarke ED, Wardman P, Wilson I. *Cis-trans* isomerization of the (5-nitro-2-furyl)acrylamide, AF-2, initiated by ascorbate, glutathione, Fe(II) and OH-. *Biochem Pharmacol* 1984; **33**: 83: 83: 7. doi: [https://doi.org/10.1016/0006-2952\(84\)90373-3](https://doi.org/10.1016/0006-2952(84)90373-3)
136. Cherdyntseva NV, Ivanova AA, Ivanov VV, Cherdyntsev E, Nair CK, Kagiya TV. Ascorbic acid glucoside reduces neurotoxicity and glutathione depletion in mouse brain induced by nitrotriazole radiosensitizer. *J Cancer Res Ther* 2013; **9**: 364–9. doi: <https://doi.org/10.4103/0973-1482.119303>
137. Zhou L, Ishizaki H, Spitzer M, Taylor KL, Temperley ND, Johnson SL, et al. ALDH2 mediates 5-nitrofurantoin activity in multiple species. *Chem Biol* 2012; **19**: 883–92. doi: <https://doi.org/10.1016/j.chembiol.2012.05.017>
138. Peterson RT. New mechanisms of 5-nitrofurantoin toxicity. *Chem Biol* 2012; **19**: 789–90. doi: <https://doi.org/10.1016/j.chembiol.2012.07.001>
139. Saulnier Sholler GL, Bergendahl GM, Brard L, Singh AP, Heath BW, Bingham PM, et al. A phase I study of nifurtimox in patients with relapsed/refractory neuroblastoma. *J Pediatr Hematol Oncol* 2011; **33**: 25–30. doi: <https://doi.org/10.1097/MPH.0b013e3181f47061>
140. Du M, Zhang L, Scorsone KA, Woodfield SE, Zage PE. Nifurtimox is effective against neural tumor cells and is synergistic with buthionine sulfoximine. *Sci Rep* 2016; **6**: 27458. doi: <https://doi.org/10.1038/srep27458>
141. Li Q, Lin Q, Kim H, Yun Z. The anti-protozoan drug nifurtimox preferentially inhibits clonogenic tumor cells under hypoxic conditions. *Am J Cancer Res* 2017; **7**: 1084–95.
142. Castro JA, de Mecca MM, Bartel LC. Toxic side effects of drugs used to treat Chagas' disease (American trypanosomiasis). *Hum Exp Toxicol* 2006; **25**: 471–9. doi: <https://doi.org/10.1191/0960327106het6530a>
143. Chen Q, Espey MG, Krishna MC, Mitchell JB, Corpe CP, Buettner GR. Ascorbic acid at pharmacologic concentrations selectively kills cancer cells: ascorbic acid as a pro-drug for hydrogen peroxide delivery to tissues. *Proc Natl Acad Sci USA* 2005; **102**: 13604–9. doi: <https://doi.org/10.1073/pnas.0506390102>
144. Doskey CM, Buranasudja V, Wagner BA, Wilkes JG, Du J, Cullen JJ, et al. Tumor cells have decreased ability to metabolize H₂O₂: Implications for pharmacological ascorbate in cancer therapy. *Redox Biol* 2016; **10**:

- 274–84. doi: <https://doi.org/10.1016/j.redox.2016.10.010>
145. Du J, Cieslak JA, Welsh JL, Sibenaller ZA, Allen BG, Wagner BA, et al. Pharmacological ascorbate radiosensitizes pancreatic cancer. *Cancer Res* 2015; **75**: 3314–26. doi: <https://doi.org/10.1158/0008-5472.CAN-14-1707>
146. O'Connor LJ, Cazares-Körner C, Saha J, Evans CN, Stratford MR, Hammond EM, et al. Design, synthesis and evaluation of molecularly targeted hypoxia-activated prodrugs. *Nat Protoc* 2016; **11**: 781–94. doi: <https://doi.org/10.1038/nprot.2016.034>
147. Bonnet M, Hong CR, Gu Y, Anderson RF, Wilson WR, Pruijn FB, et al. Novel nitroimidazole alkylsulfonamides as hypoxic cell radiosensitisers. *Bioorg Med Chem* 2014; **22**: 2123–32. doi: <https://doi.org/10.1016/j.bmc.2014.02.039>
148. Hunter FW, Wouters BG, Wilson WR. Hypoxia-activated prodrugs: paths forward in the era of personalised medicine. *Br J Cancer* 2016; **114**: 1071–7. doi: <https://doi.org/10.1038/bjc.2016.79>
149. Liu C, Lin Q, Yun Z. Cellular and molecular mechanisms underlying oxygen-dependent radiosensitivity. *Radiat Res* 2015; **183**: 487–96. doi: <https://doi.org/10.1667/RR13959.1>