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Overview

Chemical Radiosensitizers for Use in Radiotherapy☆

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ABSTRACT:
Radiosensitizers are intended to enhance tumour cell killing while having much less effect on normal tissues. Some drugs target different physiological characteristics of the tumour, particularly hypoxia associated with radioresistance. Oxygen is the definitive hypoxic cell radiosensitizer, the large differential radiosensitivity of oxic vs hypoxic cells being an attractive factor. The combination of nicotinamide to reduce acute hypoxia with normobaric carbogen breathing is showing clinical promise. 'Electron-affinic' chemicals that react with DNA free radicals have the potential for universal activity to combat hypoxia-associated radioresistance; a nitroimidazole, nimorazole, is clinically effective at tolerable doses. Hypoxia-specific cytotoxins, such as tirapazamine, are valuable adjuncts to radiotherapy. Nitric oxide is a potent hypoxic cell radiosensitizer; variations in endogenous levels might have prognostic significance, and routes to deliver nitric oxide specifically to tumours are being developed. In principle, many drugs can be delivered selectively to hypoxic tumours using either reductase enzymes or radiation-produced free radicals to activate drug release from electron-affinic prodrugs. A redox-active agent based on a gadolinium chelate is being evaluated clinically. Pyrimidines substituted with bromine or iodine are incorporated into DNA and enhance free radical damage; fluoropyrimidines act by different mechanisms. A wide variety of drugs that influence the nature or repair of DNA damage are being evaluated in conjunction with radiation; it is often difficult to define the mechanisms underlying chemoradiation regimens. Drugs being evaluated include topoisomerase inhibitors (e.g. camptothecin, topotecan), and the hypoxia-activated anthraquinone AQ4N; alkylating agents include temozolomide. Drugs involved in DNA repair pathways being investigated include the potent poly(ADP ribose)polymerase inhibitor, AG14361. Proteins involved in cell signalling, such as the Ras family, are attractive targets linked to radioresistance, as are epidermal growth factor receptors and linked kinases (drugs including vandetanib [ZD6474], cetuximab and gefitinib), and cyclo-oxygenase-2 (celecoxib). The suppression of radioprotective thiols seems to offer more potential with alkylating agents than with radiotherapy, although it remains a strategy worthy of exploration. Wardman, P. (2007). *Clinical Oncology* 19, 397–417

Key words: Chemoradiation, DNA, hypoxia, nitroimidazoles, oxygen, radiosensitizers

Introduction

In the present context, the measures of radiosensitivity of most interest are the clonogenic survival of tumour cells, and the survival of cells in, or functionality of, normal tissues, after doses of radiation delivered with therapeutic intent. Variations in these measures of radiosensitivity reflect many factors. Differences in response with radiation quality might arise from different distributions of the initial ionization events, leading to differences in the nature, yields and/or spatial distribution (especially clustering) of damage from the free radicals that are the ultimate cause of cell death or pathological change. Chemicals — oxygen is an example — can react with these free radicals and modify response. Differences in radiosensitivity might reflect variations in the levels or activity of proteins involved in the repair of damage to DNA, linked in turn to gene expression: chemicals that inactivate such proteins might be radiosensitizers. As cells progress (or not) through the cell cycle, checkpoints and signalling events may vary in their efficiencies, and can be modified by drugs.

We consider here only the modulation of radiosensitivity by low molecular weight chemicals. These can be both endogenous substances, such as oxygen, nitric oxide, thiols and ascorbate (the levels of all of which can both vary and be modulated), and xenobiotic chemicals, which interact with radiation damage in some way. They can be further separated into substances that react with short-lived free radicals and need to be present at the instant of irradiation (e.g. oxygen), and those that target radiation effects more indirectly, such as by binding to DNA repair enzymes or cell

☆ The chemical structures of many of the drugs referred to in this overview are shown in Fig. 1; these are asterisked on first mention.

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signalling proteins to render them ineffective. Radiation therapy is often given in conjunction with a course of chemotherapy; in some instances this includes regimens in which therapeutic gain is sought by exploiting synergy between radiation and drug effects. An example would be the combination of drugs that kill radioresistant hypoxic cells with a radiotherapy course. Although the planning of such a regimen would consider the two treatments in concert because the target cell population varies during the radiotherapy course because of differential radiosensitivity of hypoxic vs oxic cells, the effects are in principle independent, and this topic is not discussed here. However, some drugs may both kill hypoxic cells *and* react with short-lived, radiation-produced free radicals; these are discussed below. Furthermore, the independence of action is often a grey area: if one defines radiation effects to include a long post-irradiation period, it may be unclear whether any effects of chemotherapy given any time after irradiation are truly independent of radiobiological effects. The terminology in discussing the interaction of cytotoxic chemotherapy with radiation has long been a problem [1,2], yet 'our understanding of the specific mechanisms of interaction between radiation and chemotherapy is still evolving' [3]. The present overview cannot hope to encompass all aspects of the interaction of radiation with drugs; a recent review [4] set out the general principles of the 'concurrent chemoradiation paradigm', and previous papers have discussed the biological basis for combining drugs with radiation [3] and reviewed many trials of combined radiation/drug treatment [5]. A comprehensive overview of both radiation sensitizers and protectors showed the breadth of the topic [6]; new and emerging radiosensitizers and radioprotectors have been reviewed recently [7], focusing on the newer chemoradiation modalities. A report of a meeting to advise the International Atomic Energy Agency on radiosensitizers meriting further development also described the newer approaches [8].

Chemical radioprotectors are, of course, the reverse of radiosensitizers: the aim is to decrease radiosensitivity, especially of normal tissues. Clinical gain can be either by a reduction in morbidity if the effects are confined to normal tissues, or by exploiting the hoped-for reduced radiosensitivity of normal tissues to deliver higher radiation doses and, thus, enhanced tumour cell kill, the latter strategy obviously not without risk. The best-known radioprotector is the thiol prodrug, amifostine* (WR-2721). Activity in this field has been included in other reviews [6,7,9–12] and is not discussed in detail here. The importance of chemical radioprotectors is that their existence illustrates the competition between the enhancement of damage (e.g. by oxygen or drugs) and 'repair' in the specific example involving the reaction of short-lived free radicals with thiols, or thiol drugs [6,9].

As key discoveries in the 1970s relevant to this area are becoming less well known with time (an example is the millisecond timescale of the 'oxygen effect' [13,14]), some early landmark advances are noted, along with a brief overview of the current status. The field is too large for a comprehensive survey in this overview, and only illustrative references are given.

Types of Chemical Radiosensitizer

An early pioneer in this field, G. E. Adams, divided radiosensitizers into five categories [15,16]:

- 'Suppression of intracellular-SH [thiols] or other endogenous radioprotective substances.
- Radiation-induced formation of cytotoxic substances from the radiolysis of the sensitizer.
- Inhibitors of post-irradiation cellular repair processes.
- Sensitization by structural incorporation of thymine analogues into intracellular DNA.
- Oxygen-mimetic sensitizers, for example the electron-affinic drugs …'.

All these types of radiosensitizer are discussed below, although the order and emphasis is changed, and there is new interest in cell signalling processes and growth factors so that post-irradiation pathways of interest extend beyond DNA repair.

Another leader in this area, E. J. Hall, in discussing radiosensitizers, stressed the importance of a differential effect between tumours and normal tissue, and with this 'all important criterion' suggested in the fifth edition of his standard text [17] 'only two types of sensitizers have found practical use in clinical radiotherapy:

- The halogenated pyrimidines … based on the premise that tumor cells cycle faster and, therefore, incorporate more drug than the surrounding normal tissues.
- Hypoxic cell sensitizers increase the sensitivity of cells deficient in molecular oxygen … based on the premise that hypoxic cells occur only in tumors and not in normal tissues.'

This focus now seems too narrow to the present author, or at least 'hypoxic cell sensitizers' can now be broadened as a term far beyond the original concept. Taking up the latter premise presented by Hall (recognising that the issue is not clear-cut, with a spectrum of oxygen tensions across both tumours and normal tissues), it is pertinent to note recent progress in oxygen-sensitive drug delivery. In principle, many drugs can be specifically released only in cells of low, defined oxygen tension, exploiting the 'trigger-effector' concept, developed especially by the group of W. A. Denny and W. R. Wilson [18], and now attracting wider attention [19,20]. This approach, illustrated in Fig. 2, involves constructing prodrugs comprising a bioreducible 'trigger' (often nitroaromatic moieties based on experience with 'electron-affinic' radiosensitizers), which when reduced by cellular enzymes (donating an electron to form a radical anion), fragments to release active drug. This release can be made selective to hypoxia because the intermediate prodrug radical is oxygen reactive; oxygen inhibits drug release via a fast, free radical (electron transfer) reaction. Profiling drug release to oxygen tensions involves matching the rates (chemical kinetics) of the reactions involved [21]. A recent illustration from the author's institute shows significant

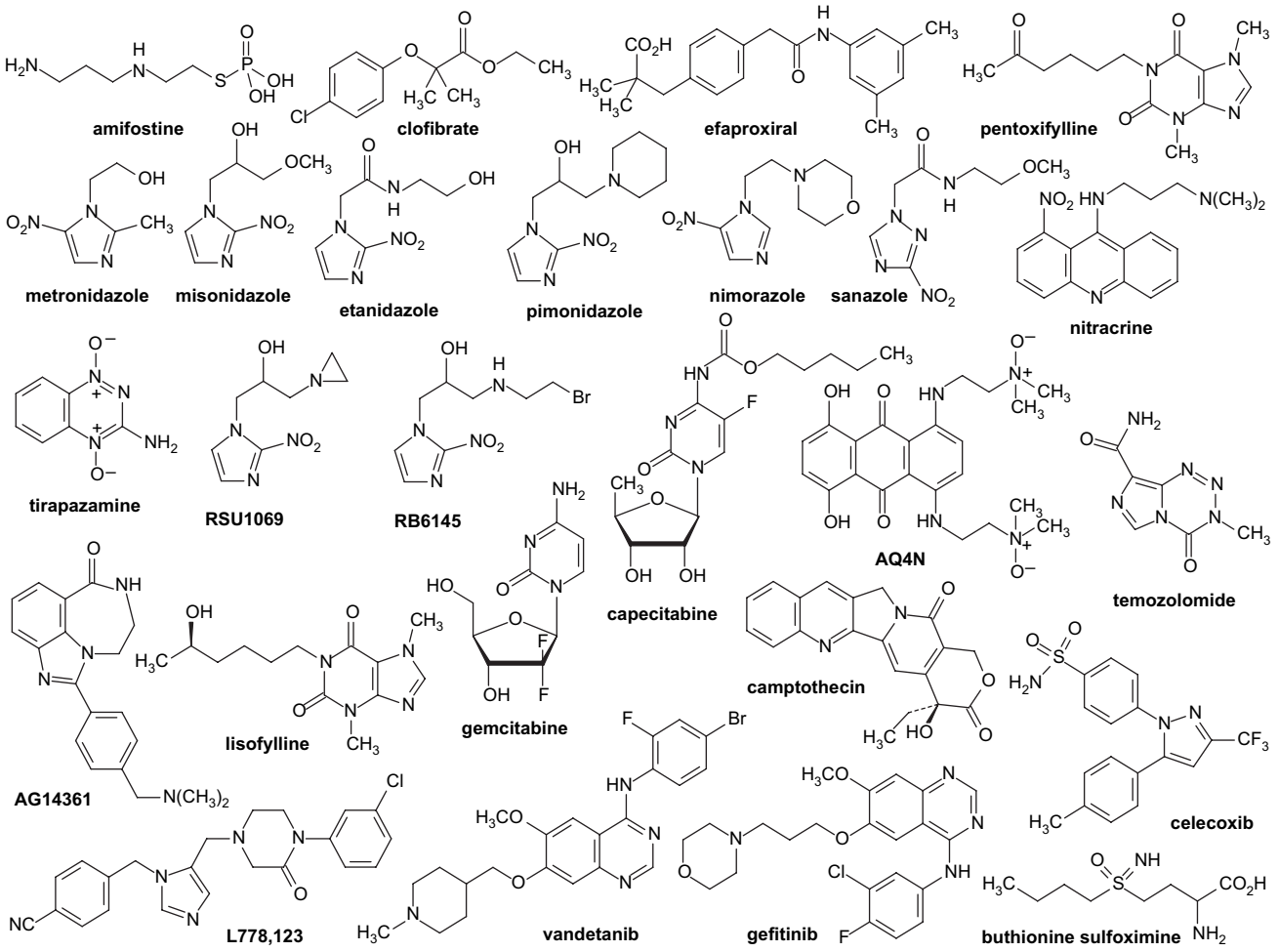

Fig. 1 — Some of the drugs discussed in the text (those asterisked at first mention).

progress in designing prodrugs having the desired characteristics [22].

Hence, it is, in principle, possible to deliver any drug that enhances cellular radiosensitivity to tissues having low oxygen tensions. The inclusion by Adams in his 1973 classification of 'Inhibitors of post-irradiation cellular repair processes' is especially relevant today because of the vastly increased understanding of DNA repair and cell signalling, and the development of potent inhibitors of these pathways. Such inhibitors need not focus on a fundamental differential between pathways in tumours vs normal tissues if drug delivery can be made tumour specific, e.g. by hypoxia-controlled targeting via oxygen-sensitive prodrug fragmentation. Of course, the pharmacological and pharmacodynamic characteristics of prodrug and drug need to be such that desirable 'bystander' effects in oxygenated tumour cells are obtained without systemic redistribution of drug thwarting initially specific delivery. There is obvious scope to apply this approach to drugs that have been used in conjunction with radiotherapy and that modulate DNA repair or cell signalling pathways.

Radiotherapy is Free Radical Therapy: the Basis for Oxygen and 'Oxygen-mimetic' Hypoxic Cell Radiosensitizers

With developments in cell biology, effort in radiobiology research has shifted down the radiation effects' timeline: one review a few years ago entitled 'How does radiation kill cells?' does not refer to free radicals at all [23], reflecting interest in targeting cell signalling pathways as an emerging and attractive therapeutic strategy. However, most insight into the use of arguably the most important class of chemical radiosensitizers can be gained by recognising that short-lived free radicals are obligate intermediates in the complex pathways leading ultimately to cell kill after radiation: '*ionizing* radiation' implies free radical production — ionisation is loss of an electron and free radicals are species with unpaired electrons. Oxygen, the prototypical radiosensitizer in many respects, is itself a free radical, but unusual in having two unpaired electrons and rapidly adding to many other free radicals, producing new, reactive radicals.

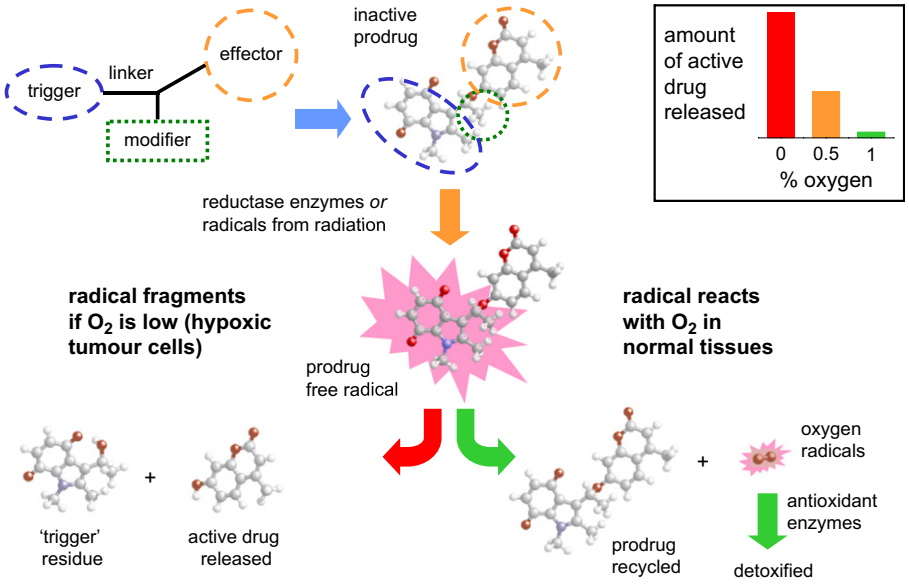

Fig. 2 — Illustration of how drugs can be delivered to hypoxic tissues using the 'trigger—effector' concept. Inactive prodrugs can be reduced either by cellular enzymes or by radiation-produced radicals, the resulting free radical then fragmenting to release active drug or reacting with oxygen to recycle the prodrug. The competition between the two pathways is controlled by the kinetics of the reactions and oxygen tension. Chemical groups ('modifiers') on the linking moiety can change the profile of active drug release to suit the desired oxygen tension targeted.

Oxygen: the Definitive Hypoxic Cell Radiosensitizer

The links between tumour hypoxia and prognosis after radiotherapy, as well as with tumour proliferation, sensitivity to some chemotherapy regimens, and the malignant phenotype, are now well established [24]. Two recent reviews discussing hypoxia in head and neck cancer [25,26], and a commentary providing a brief overview of the current understanding of tumour hypoxia [27] illustrate both the diversity of hypoxia-associated phenomena and progress towards patient profiling using diagnostic probes. The simplest representation of the role of oxygen in influencing radiosensitivity, and thiols in 'repairing' radiation damage, is often seen in equations such as: '$R^{\bullet} + O_2 \rightarrow$ damage fixation', in competition with: '$R^{\bullet}$ + thiol $\rightarrow$ damage repair', where $R^{\bullet}$ represents an unspecified free radical. These correctly suggest a competing scenario between oxygen and thiols, but tell us nothing about both the mechanisms involved ('fixing' damage is particularly uninformative and misleading) and the efficacy of oxygen, in concentration terms, as a chemical radiosensitizer. The latter is not easy to measure, even *in vitro*, because of cellular respiration and inefficient gas/liquid equilibration [28], although appropriate techniques have been developed [29].

The oxygen concentration giving a response midway between well-oxygenated and anoxic cells (with a typical maximum differential radiosensitivity of about a factor 2.5—3) is equivalent to around 0.4% v/v oxygen (gas phase) or 0.4 kPa (3 mm Hg) partial pressure (pO_2) [17], but most early measurements involved much higher radiation doses than are commonly given in fractionated radiotherapy. Although there has been much work in the last decade in characterising the form of radiation survival curves at clinically relevant (<5 Gy) doses using *in vitro* models [30], not always translating to corresponding effects *in vivo* [31], almost all of the data refer to air-equilibrated cells (which incidentally all lack ascorbate, a key 'radical sink'). There is evidence that oxygen is less efficient in radiosensitizing cells at therapeutic compared with higher radiation doses [32—34], but again studies generally compare air-equilibrated cells with anoxia and not the response at physiologically relevant (much lower) oxygen tensions. An illustration of the response measured at clinically relevant doses and oxygen tensions in one *in vitro* model in a recent study in the author's institute [35] is shown in Fig. 3. In spite of the historical data being based on high radiation doses, it is clear that many common tumours include a significant fraction of cells with intracellular oxygen concentrations in the steeply rising radiosensitivity response/concentration region, such that any method to improve tumour oxygenation should result in an increase in radiosensitivity of hypoxic subpopulations. The potential gain of radiosensitizing hypoxic cells is large: severely hypoxic cells typically require two to three times higher radiation doses to kill them compared with well-oxygenated cells [17], a factor independent of absolute radiosensitivity across organisms differing in radiosensitivity by orders of magnitude.

The most direct, and perhaps the earliest, treatment for raising tumour oxygen levels is hyperbaric oxygen: clinical trials have been well documented, most benefit being seen with few/large fraction radiation regimens [36,37]. The method is cumbersome and with some increase in morbidity

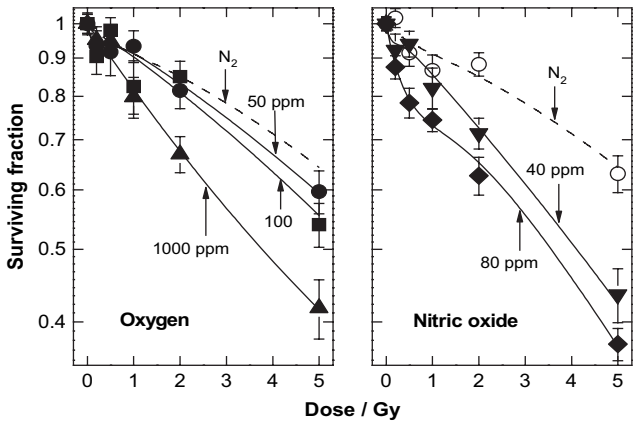

Fig. 3 — Radiosensitization of hypoxic Chinese hamster V79-379A fibroblast-like cells *in vitro* by either oxygen or nitric oxide at very low concentrations (gas phase ppm v/v) and low radiation doses; clonogenic survival data from [35]. Anoxic (N_2) (open circle); 50 ppm O_2 (solid circle); 100 ppm O_2 (solid square); 1000 ppm O_2 (solid up triangle); 40 ppm nitric oxide (solid down triangle); 80 ppm nitric oxide (solid diamond). Lines are drawn to guide the eye; the dashed line is the response in anoxia (data points are omitted from the left-hand panel for clarity). Although cellular consumption might cause the efficacy of oxygen to be underestimated, nitric oxide seems to be more potent than oxygen (NB nitric oxide is ~50% more soluble in media than oxygen for the same partial pressure).

in some sites [36]. Although hyperbaric oxygen is not in use today with radiation, its use to treat late radiation damage is attracting attention [38]. Oxygen carriers, mainly based on enhanced solubility parameters of perfluorocarbons compared with blood plasma, have been evaluated, with mixed results; thus one recent animal study [39] found benefit only when the perfluorocarbon was combined with carbogen breathing (carbogen is usually 95—98% O_2 + 5—2% CO_2 v/v). However, newer approaches are being developed, exploiting advances in nanotechnology [40], and there are reasonable prospects for the development of new oxygen carriers that might have value in radiotherapy.

Other approaches to increase the oxygen supply to the tumour have been attempted; strategies to modify oxygen transport via haemoglobin binding look particularly interesting. The effect of anaemia on radiation therapy has been recently reviewed [41—43]; a comparison with animal studies has been made [44]. Although the 'literature ... is overwhelmingly of its importance as a prognostic factor' [36], both the most recent [43] and an earlier discussion [36] noted the difficulty in defining optimal haemoglobin levels. Transfusion with red blood cells often seems to be limited to cases of severe anaemia [43]; it may be detrimental in some instances because of immunosuppression [42,43]. A paper [45] discussed the interrelationships of low haemoglobin levels, hypoxia, tumour angiogenesis and survival. Derivatising haemoglobin with polyethylene glycol to improve the biocompatibility of bovine haemoglobin was found to be useful in animal models involving fractionated radiation, particularly in carbogen-breathing mice [46], but clinical studies are awaited. Recombinant human erythropoietin has been evaluated clinically in some detail as a means to correct haemoglobin levels [43], but its many functions have been noted, including its angiogenic and mitogenic potential [42]. Reviewing published studies, Kaanders *et al.* [47] concluded that 'The potential of erythropoietin to promote tumor growth must be further investigated, but it is too early to withdraw erythropoietin from the clinic for this reason'. Indeed, a recent evaluation [48] suggested: 'Most studies suggest that erythropoietic therapy either improves survival or has no negative effect on survival when used to treat anaemia in patients with cancer ... When used according to its licensed indications, it is likely that erythropoietic therapy has no negative effect on survival.'

Clofibrate* is an anti-lipidaemic drug that reduces the affinity of haemoglobin for oxygen and thus acts as a radiosensitizer [49], but clinical studies in this context do not appear to have been carried out. Efaproxiral* (RSR13) also interacts with haemoglobin in a non-covalent and allosteric manner to lower the oxygen-binding affinity, and increase pO_2 [50—52]. A number of clinical trials are in progress or have been completed [46,53—55]. A phase III trial of efaproxiral, involving 515 patients treated with whole-brain radiotherapy for brain metastases, showed a significant increase in response rate for the efaproxiral arm at 3 and 6 months, with evidence that patients with breast primary tumours responded better [55].

Pentoxifylline* has been reported to improve tumour oxygenation and improve radiation response if given before irradiation in several animal studies (e.g. [56]); its modes of action include increased red cell deformability, reduced blood viscosity as a result of decreasing platelet aggregation, and vasodilation. Reviewing the evidence, however, Nieder *et al.* [57] considered '... these findings have not translated into positive clinical studies to date. None of three published clinical trials attempting to enhance the effectiveness of radiotherapy with Ptx [pentoxifylline] had a satisfactory design'.

Nicotinamide was originally evaluated in radiobiology as an inhibitor of DNA repair via its interaction with poly(ADP ribose) polymerase (PARP), as an analogue of known PARP inhibitors, but has found more value as a vasoactive agent. It showed good activity in combination with carbogen or normobaric oxygen with fractionated irradiation in animal models [58], where it was shown to eliminate acute hypoxia (intermittent closure of blood vessels) [59]. The possibility of repair inhibition in normal tissues *in vivo* does not seem to be a problem [60], and although it was suggested that small dose reductions might be needed because of some normal tissue sensitization [61], several promising clinical studies have been reported. These mainly involve the combination of nicotinamide with normobaric carbogen (to overcome diffusion-limited or chronic hypoxia), often with accelerated radiotherapy to inhibit repopulation (the 'ARCON' regimen: accelerated radiotherapy with carbogen and nicotinamide) [36,62,63]. A phase II study of bladder cancer found no difference in bowel and bladder morbidity at 12 weeks, although there were problems with patients

completing daily nicotinamide at 80 mg/kg over 20 fractions/4 weeks [64]; another study discussed possible relationships between tolerance and pharmacokinetics [65], and daily doses of 60 mg/kg became normal. Acute and late morbidity in the treatment of advanced bladder carcinoma with the ARCON regimen was assessed again more recently [66], concluding that 'Although, for some endpoints, the incidence of late sequelae was higher than expected, overall morbidity was no worse than reported by others. The data indicated that ARCON could achieve a therapeutic gain in patients with advanced bladder carcinoma'. Reviewing ARCON in 2002, Kaanders *et al*. [67] considered 'In particular in cancers of the head and neck and bladder, the local tumour-control rates are higher than in other studies …'; another review [47] confirmed this conclusion, although in unselected groups of patients in a phase I/II study there was no significant difference in tumour response and local control with carbogen and nicotinamide added to conventional radiotherapy [68]. ARCON treatment reduced the prognostic significance of haemoglobin in squamous cell carcinoma of the head and neck [69]. The outcome of phase III trials is awaited with interest.

'Electron-affinic' Radiosensitizers: the Nitroimidazoles and Related Drugs

In the 1960s, comparison of the chemical properties of chemicals that radiosensitized anoxic cells with their reactivity towards radiation-produced free radicals led to the identification of an important class of 'electron-affinic' radiosensitizers [15,16,70]. Early prototypes were nitrobenzenes [71,72], but practical drugs based on nitroimidazoles, such as metronidazole*, already in clinical use as trichomonacidal agents, soon emerged [73]. The best known of these as radiosensitizers is misonidazole* (Ro 07-0582), which had activity in all solid murine tumour models tested (to the author's knowledge): a compilation in 1981 listed almost 50 studies [74], involving end points such as local control, regrowth delay and cell survival. No sensitization of normal tissues was generally found, but mild hypoxia (i.e. sensitization) in skin [75] and effects in mouse tail tissue were shown [76]. It was expected [16], and found [74,77], that efficacy was reduced (but not eliminated) in multi-fractionated treatments because of re-oxygenation between treatments. Cells at intermediate oxygen tensions are especially critical in defining the effects of hypoxic cell radiosensitizers [78].

However, in the event, toxicity in humans was dose limiting: the efficacy of misonidazole as a radiosensitizer was not proven after numerous trials [36], although a meta-analysis involving trials including over 7000 patients indicated significant benefit of locoregional control after radiotherapy given with nitroimidazole radiosensitizers [79]. Toxicity *in vitro* paralleled radiosensitization efficacy, because the same property — electron affinity or reduction potential — dominated the structure—activity relationship [80,81]. Attempts to improve the therapeutic ratio *in vivo* by increasing hydrophilicity (to decrease peripheral neuropathy), as in desmethylmisonidazole (Ro 05-9963) and etanidazole* (SR2508) were not as successful as hoped [9,82]. Pimonidazole* (Ro 03-8799 [83]) showed enhanced efficacy *in vitro* in (extracellular media) concentration terms compared with misonidazole without correspondingly enhanced cytotoxicity [83], but this may have reflected pH gradients between culture media and intracellular milieu [84], or between intracellular organelles [85] (the compound is a fairly strong base with $pK_a \sim 8.7$ compared with nimorazole's $pK_a \sim 5.0$). Despite good tumour uptake [86], pimonidazole, too, was not found to be effective in trials [36], but it was 'reborn' as a useful diagnostic probe for hypoxia [87].

However, drugs were revisited that had been 'passed by' because of, with hindsight, over-optimistic expectation of realising high dose-modifying effects. There is evidence from the redox dependence of radiosensitization efficiency with electron affinity, which varies with the level of response analysed [80], that there is more than one mechanism of radiosensitization. Compounds with low, but still significant, efficacy *in vivo* could be tolerated to very high doses. In particular, the 5-nitroimidazole, nimorazole* was shown to be effective in several clinical trials [37,79], and it has been in routine use in the treatment of head and neck cancer in Denmark. It is most unfortunate that its 'orphan drug' status seems to inhibit its wider use despite (or because of?) the cheapness of the treatment. It is clear that the better selection of patients for inclusion in trials involving treatments aimed at hypoxic cells would be beneficial. In the context of nimorazole, for example, high concentrations of osteopontin in plasma were related to the response seen with nimorazole in the Danish DAHANCA 5 trial [88].

Numerous alternative nitroaromatic structures were evaluated as alternatives to misonidazole and its nitroimidazole analogues [89], of which the nitrotriazole, sanazole* (AK-2123), is the most enduring [90]. A large (462 patients) phase III clinical trial of sanazole in the treatment of squamous cell carcinoma of the uterine cervix showed an increase in local tumour control and survival without the addition of any major toxicity [91]. (A criticism of many of the early nitroimidazole trials was that they were too small.) An interesting development of nitroaromatic chemistry involving sanazole is its evaluation as a possible radiopharmaceutical agent designed for therapy rather than imaging: a derivative of sanazole linked to a chelating agent complexed with ^{177}Lu has been described [92]. (Nitroaromatic compounds bind selectively to hypoxic cells via complex reductive chemistry involving an intermediate, oxygen-sensitive radical: numerous studies aimed at diagnosing hypoxia and involving immunohistochemical [87,93,94] or radiopharmaceutical [95] detection have been carried out with nitroimidazoles.) Doranidazole (PR-350) is a nitroimidazole very similar to misonidazole but with greater hydrophilicity; a phase III trial, involving only 48 patients, of doranidazole combined with intraoperative radiotherapy in advanced pancreatic cancer, did not show significant gain [96].

The nitroimidazoles and related compounds are often termed 'oxygen-mimetic' radiosensitizers. There are some parallels in their reactivity towards DNA base radicals that

might justify this description. Fig. 4 shows *possible* mechanisms by which both oxygen and nitroimidazoles and related compounds might enhance DNA strand breaks, based on many radiation—chemical studies [97—100]. A key, but now often overlooked, set of experiments was carried out in the 1970s, which showed that both types of radiosensitizer had to be present at the instant of irradiation: adding either a few milliseconds after irradiation was ineffective [13,14]. This supports a free radical mechanism and should steer investigators away from more 'modern' explanations involving effects of hypoxia on cell signalling pathways, which seem unlikely to be able to be 'switched' on or off on this timescale. An important property of the 'electron-affinic' class is that they appear to radiosensitize hypoxic cells but have no measurable effect in well-oxygenated cells, at least *in vitro* (any small effects on normal tissues [75] probably reflect tissue oxygen levels much lower than those commonly encountered in *in vitro* models). This is probably not because of some remarkable inherent property, but because of simple kinetic competition between oxygen and nitroimidazole (or analogue) for reaction with key DNA base radicals. The competition is characterised by the product of reactivity (chemical rate constant) and concentration: the high reactivity of oxygen ensures it out-competes the radiosensitizer. This begs the question as to the probable competition if drugs are designed to bind to DNA, e.g. by intercalation or electrostatic interaction. The topic of DNA targeted radiosensitizing drugs has attracted attention [101]. Examples include nitroimidazole/intercalator conjugates [102,103], nitroacridines (e.g. nitracrine*) and nitroquinoline intercalators [104], minor groove binders [105], and polyamine conjugates [106]. Even pimonidazole was shown to be 'concentrated' near DNA relative to misonidazole [107,108]. However, diffusion-limited hypoxic cells are distant from capillaries, so free diffusion unimpeded by binding to cells nearest capillaries is a desirable property, and this targeting approach does not appear to have been useful clinically to date. On the issue of diffusion of drug from the vasculature to distant hypoxic cells, it is notable that significant advances in understanding the processes influencing drug delivery and distribution in tumours have been made as a result of interest in this issue with radiosensitizers and hypoxic cell toxins [109—112]. This experience is highly relevant to cancer treatment by drugs in its widest sense.

Aromatic N-oxides: Alternatives to Nitroaromatic Compounds with Most Value as Hypoxic-specific Cytotoxins

Some aromatic *N*-oxides, especially the benzotriazine di-*N*-oxide, tirapazamine* (SR4233), have attracted considerable attention, including several clinical trials, because of selective cytotoxicity towards hypoxic cells in the absence of radiation [24,113,114]. This is a property also shared with nitro compounds — metronidazole is prototypical

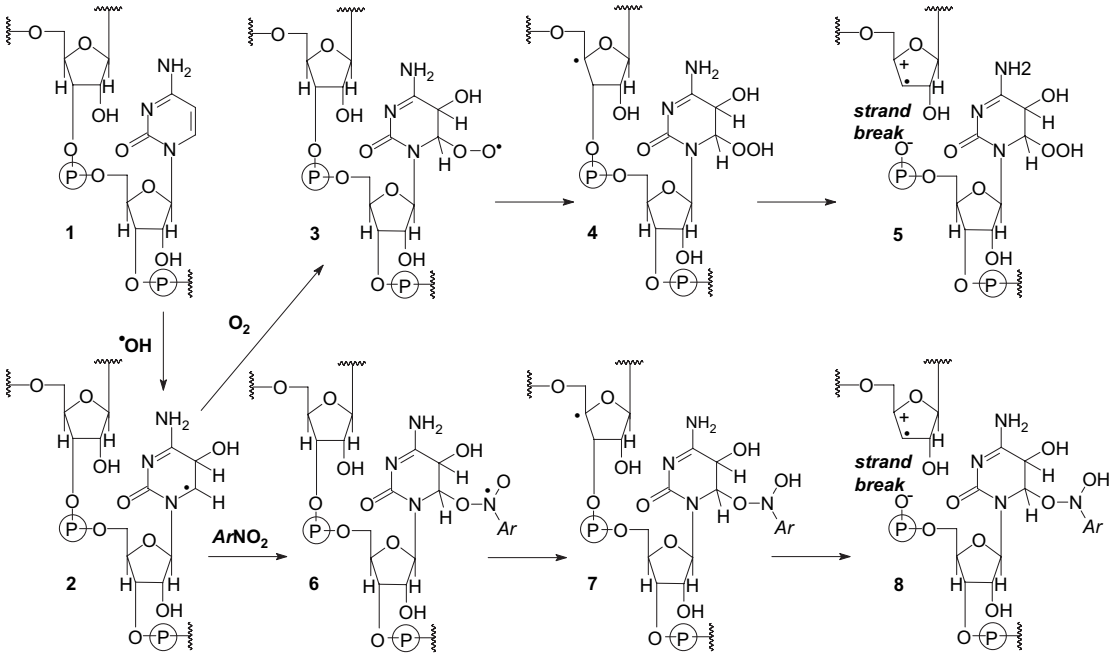

Fig. 4 — Possible pathways by which hydroxyl radicals (•OH) can add to the 5,6-double bond of pyrimidines (1) to form a carbon-centred radical (2) that can either add oxygen to form a peroxyl radical (3) or add to the oxygen atom in the nitro moiety of a nitroaromatic radiosensitizer ($ArNO_2$) to form a radical adduct (6). In either case the intermediate radical (3) or (6) might abstract hydrogen from a neighbouring sugar C—H bond (5′ in this example, although 3′-abstraction may occur) to transfer radical damage from base to sugar (4) or (7), leading to a strand break (5) or (8). Based mainly on a scheme by Bamatraf *et al.* [100] and extensive studies by other workers described by von Sonntag [97,98]. Only part of the overall mechanism of strand break formation is shown; for further details see [98].

[115], if inefficient — but to a less marked degree. Both types of compound rely for their selectivity as hypoxia-specific cytotoxins on the fast reaction between drug radicals and oxygen [116,117]. However, whether benzotriazine-*N*-oxides, for example, can also act as hypoxic cell radiosensitizers in a similar manner to oxygen or nitroimidazoles (either phenomenologically or mechanistically) is less certain, although it seems probable. The only unambiguous method to answer this point would be to conduct rapid-mix experiments where both pre- and post-irradiation exposure are so short that any effects arising from hypoxic metabolism to reactive radicals can be discounted: oxygen and nitroimidazoles do act on a very short exposure timescale [13,14]. There is absolutely no question that tirapazamine potentiates cell killing with fractionated irradiation, but activity can be measured if tirapazamine is given *after* radiation and the data 'can be largely accounted for by complementary cytotoxicity of the two agents' (drug and radiation) [118]. Notwithstanding the fact the chemical mechanisms of radiosensitization by nitroaromatics, let alone *N*-oxides, are not well established, *if* sensitizer efficiency for the *N*-oxides followed the same relationship between electron affinity as does nitroaromatics [80], then tirapazamine would be intermediate in efficiency between misonidazole and metronidazole, as its reduction potential is close to midway between these nitroimidazoles [119]. Certainly another chemical property that might be suggestive — the reactivity of the drug radical anions towards oxygen — puts the aromatic *N*-oxides firmly on the same redox 'line' as nitroaromatics [120]. As such, considerably higher concentrations of tirapazamine might be needed to show significant 'conventional' radiosensitization than used in most experiments, because of the high potency as a hypoxic cytotoxin. Aerobic radiosensitization after hypoxic pre-incubation is clearly linked to drug metabolic activation [121,122]. Descriptions of related *N*-oxides as 'hypoxic cell radiosensitizers' are being made [123], but should be viewed in the context of these difficulties in interpretation. These comments should not detract from the potential value of *N*-oxides as hypoxic cytotoxins, which are obvious and of clinical importance.

Radiosensitizers that are both 'Electron-affinic' and Efficient Hypoxia-specific Cytotoxins

'Dual-function' radiosensitizers, where the drugs can act like misonidazole in modifying free radical damage, and also include a second cytotoxic functionality such as alkylating or binding, activated metabolically (and possibly radiolytically), are another class of agent where the basis for activity is not always easy to separate. The best known of these are the 2-nitroimidazoles with an aziridine moiety on the sidechain, such as RSU1069*, or the prodrug for this compound activated hydrolytically, RB6145* (CI-1010) [124—127]. Enzyme-catalysed reduction of the nitro group in hypoxic cells positively shifts the pK_a of the aziridine nitrogen in the sidechain of RSU1069 to activate the alkylating function. Clearly, this will also occur to some extent even if, for example the drug adds to radiation-produced DNA base radicals to form a radical adduct (*cf*. Fig. 4) (the pK_a of the piperidine nitrogen in pimonidazole increases from ~8.7 to ~9.2 even in the radical anion [120]). Hence, the possibility of radiation-induced cross-linking of DNA as a mechanism needs to be considered, as well as 'simple' potentiation by enzyme-catalysed reduction. A recent pre-clinical study [128] compared the efficacy of tirapazamine and RB6145 in reducing metastatic dissemination after treatment of the primary tumour with radiation and drug in a fractionated regimen, providing evidence that targeting hypoxic cells in primary tumours is a valuable strategy to reduce disseminated disease.

Nitric Oxide: the Born-again Radiosensitizer

Nitric oxide is included here as 'oxygen mimetic' as it shares with oxygen its properties of being a free radical in the 'stable' form and highly reactive towards many other free radicals. However, the similarity ends there. Although it reacts rapidly with hydrated electrons produced when water is irradiated (along with many other chemicals) [129], it has a low electron affinity on the same scale of measuring oxygen or the nitroimidazoles (reduction potential −0.55 V at pH 7 [130], lower than that of metronidazole [−0.50 V]). More importantly, the unpaired electron is able to pair up with that in, for example DNA base radicals, forming, unlike DNA base peroxyl radicals from oxygen, a non-radical species. This is unable, for example, to perform the hydrogen abstraction from a sugar, which can lead to strand breaks as shown for oxygen and nitroimidazoles in Fig. 4. A stable adduct can be separated by high performance liquid chromatography in irradiated solutions of uracil and nitric oxide [35]. In spite of this key difference, nitric oxide enhances the yields of DNA double-strand breaks in irradiated cells [35], as do oxygen and nitroimidazoles [131] (but note: there is not always correlation between double-strand breaks and clonogenic survival [132]). The mechanism in the case of nitric oxide is not at all clear, but might involve, for example, excision of the damaged base (which could perhaps be proven using cells deficient in base excision repair proteins); the repair times in the popular γ-H2AX assay seemed longer for cells irradiated in nitric oxide compared with cells irradiated in air or anoxia [35], although further work is needed to verify this.

The most remarkable property of nitric oxide as a hypoxic cell radiosensitizer is its efficiency. It was shown to be an active radiosensitizer 50 years ago [133,134], but resurrected by Mitchell *et al*. [135—137] in timely work after the discovery of its other remarkable property in the maintenance of vascular tone [138]. Recent studies by the author's colleagues, illustrated in Fig. 3, have shown the effects on cell survival *in vitro* using clinically relevant radiation doses, of only 40 ppm v/v nitric oxide (~70 nM), similar to that used in inhaled nitric oxide therapy for respiratory conditions [35]. In this study, at low radiation doses, nitric oxide seemed to be significantly more efficient than oxygen

as a hypoxic cell radiosensitizer. The rapid removal of nitric oxide by reaction with red blood cells maintains a low steady-state concentration *in vivo*. However, the potential for radiosensitization by nitric oxide by elevating its synthesis or delivery *in vivo* has been shown in a series of studies involving gene therapy [139—143], and others using a chemical source of nitric oxide [144] or physical stimulus [145].

Measurements of nitric oxide levels in human tumours are lacking — clinical measurements of tumour oxygenation became available only decades after the discovery of its importance — but one study using a microelectrode in the B16 melanoma model [146] suggested levels *much* higher than shown to enhance anoxic cell radiosensitivity *in vitro* in our recent work. This raises two hypotheses worthy of addressing: first, variations between individual patients in levels of nitric oxide in tumours have clinical, prognostic significance in radiotherapy; second, giving nitric oxide by inhalation could be useful in the radiotherapy of lung tumours.

There could be an immediate obstacle to developing nitric oxide delivery, or *in situ* generation by either giving prodrugs for nitric oxide or enhancing nitric oxide synthase using gene therapy or other protocols. This is the existence of the 'steal effect', by which systemic blood pressure reduction can make the tumour *more* hypoxic, reported in studies of the effects of a nitric oxide prodrug in rat tumours [147], and discussed in a recent review of nitric oxide in the context of tumour biology [148]. However, there are two counter-claims. First, in the case of inhaled nitric oxide, there are reports of the selectivity of vasodilation to the lung [149]; systemic vascular resistance was not reduced in mice [150]. Second, nitric oxide enhancement by gene therapy actually works in a murine tumour model of fractionated radiotherapy [143].

Notwithstanding the 'steal effect', one might have considered nitric oxide therapy as a route to improve oxygen delivery for radiosensitization, via effects on vascular tone. Paradoxically, a recent clinical study [151] showed that the *inhibition* of nitric oxide synthase by pharmacological intervention resulted in a *reduction* in tumour blood volume that could have therapeutic significance: there is much current interest in targeting the tumour vasculature [152,153]. Whether such approaches have their main role in chemotherapy rather than radiotherapy remains to be seen; Denekamp [154] commented on the limited role of vascular-mediated injury in tumour response to radiotherapy, but this pre-dates recent observations of substantial and selective damage to tumour vasculature with some treatments. There are several possible effects of nitric oxide on treatment and prognosis in cancer [148,151,155,156], and this is an area meriting further study. Effects can be indirect; thus, radiosensitization by insulin treatment was ascribed to an increase in tumour pO_2, not caused by increased tumour blood flow but ascribed to a decrease in oxygen consumption caused by nitric oxide [157]. OM-174, a synthetic analogue of Lipid A, the endotoxic principle of lipopolysaccharides, was shown to radiosensitize EMT-6 tumour cells *in vitro* via activation of the interferon-γ pathway and induction of nitric oxide synthase [158].

Radiation-induced Delivery of Cytotoxic Substances

This approach to radiosensitization exploits the increasingly sophisticated targeting of radiation delivery in a most direct manner, using radiation—chemical reactions to deliver cytotoxins to the irradiated volume. It has received limited attention. One problem is that the bulk of the radiation energy is absorbed in the bulk of the material comprising the cell (water), generating initially less than 0.3 μM reducing radicals per gray dose (mainly hydrated electrons) and the same amount of oxidizing radicals (mainly hydroxyl radicals). Both species are promiscuous in their reactivity and it is difficult to intercept their reactions, except by, often, unrealistically high concentrations of drugs ('scavengers'). Hence, if, for example, the approach is to use radiation to liberate a cytotoxin from a prodrug in the same manner as the 'trigger—effector' concept successfully applied in hypoxia-selective, enzyme-activated prodrug fragmentation (see above), then the cytotoxin must be very potent indeed. Nonetheless, there could be some selectivity to hypoxic cells if reductive activation is considered, via electrons directly or reducing radicals formed on reaction with hydroxyl radicals with amino acids (for example), as oxygen reacts rapidly with both [97,98]. As with 'electron-affinic' radiosensitizers, redox properties are critical as superoxide radicals can be reducing.

One group has carried out quite extensive studies, mainly *in vitro*, involving two types of redox switch. The first approach seeks to exploit the well-known rapid fragmentation of nitroaromatic radical anions substituted with 'leaving groups', the simplest of which is halogen [159], the practical examples in this instance being a quaternary nitrogen centre in conjugates of nitroaromatics and nitrogen mustards [160]. The chemistry proved rather complex, with great sensitivity to the nitroarene 'trigger' [161]. The second approach explores the potential of cobalt(III) as the redox target for radiation-produced reducing radicals, exploiting the known rapid fragmentation of cobalt(II) complexes [162,163]. In the latter study, the drug released on radiolytic activation was a potent DNA minor groove alkylating agent, a ring-opened analogue of CC-1065. Although both approaches are well-founded mechanistically, exemplification *in vivo* is crucial.

Another group evaluated the application of radical fragmentation after capturing radiation-produced reducing equivalents, using conjugates of indolequinones and 5-fluoro-2′-deoxyuridine [164], or the latter bearing a 2-oxo-alkyl substituent as an electron-attracting centre [165]. Significant effects of the latter approach were not shown *in vivo* using a regrowth delay assay. The very low electron affinity of an oxo-alkyl centre (cf. acetone or acetophenone) means that the latter type of prodrug has to rely on capturing electrons for activation; whereas all the other

studies also seem to focus on (hydrated) electron capture, this emphasis is probably misplaced, as a wide variety of reducing radicals formed via oxidative damage can reduce nitroarenes, cobalt(III) complexes, or indolequinones.

Other redox metals have been considered as radiosensitizers. Copper(II) complexes were first studied many years ago [166,167], and interest recently resumed [168]. The use of such redox-active complexes is paved with difficulties to investigate *in vitro* because of the well-known artefacts that can arise from reactions of the metal with hydrogen peroxide produced in the irradiated media, or with thiols in the media as well as in the cells, and the diverse roles of copper in redox biology [169,170]. Although copper complexes are feasible drugs [171], until there is clear *in vivo* exemplification, this approach seems a long way from clinical interest.

A problem generally in this area is that most *in vitro* models involve irradiating cells in a large volume excess of medium, usually containing drug unless intracellular: extracellular concentration gradients are very high. Radiation–chemical events in the medium may produce a large excess of released drug that can diffuse into cells and amplify damage, a scenario that cannot be extended to anything like the same extent *in vivo*. This lesson is also pertinent to radiosensitizer (and general drug) research in a wider sense. Early experience with thiol-reactive nitroimidazoles [172] showed the artefacts in extrapolation that can arise when a huge molar excess of reagent compared with target (e.g. cellular thiols) is involved in *in vitro* models. Indeed, this author suggested that neglect of this factor — which he termed 'simple arithmetic' as it can be done on the back of a postage stamp, let alone an envelope [173] — may be responsible for many false leads in drug research. The principle of this arithmetic test for the potential lack of translation of effects *in vitro* to reality *in vivo* is illustrated in Table 1. Basically, adding very low concentrations of (in this example) a thiol-reactive chemical to cells in dishes wipes out all the protective thiols, while still leaving plenty of drug to radiosensitize, but the effect will never be realisable *in vivo* because the arithmetic does not add up: the moles of drug cannot compete with the moles of endogenous thiol.

Gadolinium(III) is a redox metal used as a contrast agent in magnetic resonance imaging. Porphyrin-like complexes ('texaphyrins') of gadolinium(III) localise in tumours, and have been investigated as radiosensitizers (including clinical trials), although the basis for these is controversial [174,175]. The lead compound is motexafin gadolinium (PCI-0120). The complexes are reactive towards reducing radicals, electrochemical studies revealing a reduction potential of ~ -0.08 V on the same scale as other radiosensitizers [174,176]. This value seems on the high side for redox cycling (reducing oxygen to superoxide/hydrogen peroxide) [177], as the corresponding reduction potential for oxygen (1 M) to superoxide is -0.18 V [178,179]. In any case, the suggestion [177,180] that sensitization to radiation by motexafin gadolinium arises from increasing oxidative stress via redox cycling should be viewed with caution. Other 'electron-affinic' radiosensitizers redox cycle rather efficiently, but the process is clearly not responsible for their efficacy; more probably, some pathological responses are linked to the phenomenon [21]. It was noted elsewhere that cells normally generate as much oxidative stress (superoxide) in about 20 s as is produced by 1 Gy irradiation [181] — although, of course, mitochondrial production is highly compartmentalized, whereas radiolytic generation is not. The reaction of motexafin gadolinium with cellular reductants, including ascorbate and glutathione [177], which will be accompanied by superoxide formation (copper(II) behaves similarly), should be viewed in the context of the potential difficulties noted above in extrapolation of phenomena seen *in vitro*. Ascorbate was shown to be an important enhancer of effects *in vitro* [182], including DNA damage [183]. The absence of ascorbate might explain in part the negative results in some studies [175], but some effect on tumour regrowth delay with combined gadolinium/radiation treatment has been reported [184]. It is not clear whether this effect arises from 'direct' radiosensitization or enhancement in tumour oxygenation [185]. A recent review suggests 'the molecular target … appears to be thioredoxin reductase' [186]. Clinical trials with motexafin gadolinium have been summarized [54,186,187], most involving the treatment of brain metastases [188]. In phase III studies, an improved time to neurological progression in patients with brain metastases receiving whole brain radiotherapy with gadolinium has been reported. Overall, the mechanistic basis for the action of gadolinium complexes as radiosensitizers is not entirely clear — it seems doubtful whether it should indeed be classified under this section as delivering cytotoxic substances — and further studies are required to demonstrate the potential of this approach.

Porphyrins have long been used as photosensitizers in photodynamic therapy [189], but also have radiosensitizing properties; limited clinical trials with radiotherapy are being conducted [190–192]. The most extensively studied example in photodynamic therapy is probably Photofrin II®, a complex mixture of porphyrins concentrated in, or retained by, some tumours relative to some normal tissues. Their efficacy as radiosensitizers, even *in vitro*, is quite dependent on the tumour cell line [191], in contrast to radiosensitizers such as misonidazole. The mechanism underlying radiosensitization is completely unknown, but

Table 1 – Thiol depletion *in vitro* and *in vivo* after systemic administration of 1 μM or up to 3 g, respectively, of a thiol-reactive chemical with molecular weight 200 [173]

	In vitro (e.g. fibroblasts)	*In vivo* (e.g. humans)
Cell density	$\sim 10^4$–10^5/cm^3	$\sim 5 \times 10^8$/cm^3
Free thiols	~5 fmol/cell	~2–5 mmol/kg
Thiol-reactive reagent administered (example)	~1 nmol/cm^3 media ~100–10 fmol/cell	< 3 g <0.2 mmol/kg
Thiol depleted	100%	<10%
Reagent depleted	~5–50%	100%

in view of the established use of these agents in photomedicine, further work is certainly justified.

Radiosensitization by Halogenated Pyrimidines

This approach can be divided into two distinct categories, essentially distinguished by halogen: bromine/iodine vs fluorine. First, cells undergoing DNA synthesis cannot distinguish efficiently between thymidine and halogenated analogues such as bromo- or iodo-deoxyuridine (UdR) (the methyl group of thymine is about the same size as bromine or iodine atoms). If cells are treated with BrUdR or IUdR for a sufficiently long period before irradiation, significant incorporation into DNA occurs. The halogen moieties act as electron 'sinks' on irradiation, the carbon—halogen bond breaking on electron attachment to liberate free halide and form a carbon-centred free radical. This can add oxygen to form a peroxyl radical and carry out similar strand-breaking reactions as the DNA base/hydroxyl radical adduct illustrated in Fig. 4. There is a correlation between BrUdR incorporation, DNA strand breaks and clonogenic survival after irradiation [193]. Skin phototoxicity is a problem with BUdR, but not with IUdR. Although success in phase III clinical trials has been elusive, the potential application of this approach in (low dose rate) brachytherapy is of particular interest; a review of the laboratory and clinical studies to 2001 summarised both positive and negative results [6]. More recent clinical attention has focused on the iodinated analogue, and particularly on a prodrug, 5-iodo-2-pyrimidinone-2′-deoxyribose, which is converted to IUdR by an aldehyde oxidase in the liver [194]. It was suggested that this approach is suitable for drug-resistant, DNA-mismatch repair-deficient (as well as repair-proficient) tumours; IUdR and BrUdR accumulated at much higher levels in mismatch repair-deficient cells [195].

More recent approaches to the use of radiosensitizing nucleosides have focused on fluorine analogues, especially 5-fluorouracil (5-FU), 5-FUdR, gemcitabine* (2′,2′-difluoro-2′deoxycytidine), and a rationally designed prodrug of 5-FU, capecitabine* [196,197]. The latter exploits the differential activity of thymidine phosphorylase in tumour compared with normal tissue in the final of three steps in converting prodrug to active 5-FU; it was suggested [198] that capecitabine 'has the potential to replace bolus or continuous infusion of 5-FU as the standard treatment [in chemoradiotherapy] for rectal cancer' (oral 5-FU has unpredictable bioavailability [197]). Another paper reviewed the use of capecitabine and other agents as radiosensitizers in rectal cancer [199]. The mechanisms of the radiosensitizing effects of the fluorinated analogues are clearly completely different to the bromo- and iodo-nucleosides, presumably reflecting at least in part the different 'leaving-group' abilities of the halogens and van der Waals' radii (atomic size), as well as (of course) differences from substitution of halogen in base or sugar. The October 1997 issue of *Seminars in Radiation Oncology* includes 10 papers discussing relevant aspects of fluoropyrimidines; a more recent review [200] updates these. Discussing possible mechanisms, McGinn and Lawrence [197] noted that 'Incorporation of … BrUrd and IdUrd into DNA has been associated with increased induction and decreased rate of repair of radiation-induced DNA damage. Studies using similar techniques have found no effect on radiation-induced DNA damage or repair after exposure to gemcitabine under conditions known to produce radiosensitization. … In contrast, data tend to support the hypothesis that gemcitabine-mediated radiosensitization is related to concurrent disruption of deoxyribonucleotide pools and redistribution of cells into S phase of the cell cycle'. (Cells are usually most radiosensitive close to mitosis, and most radioresistant in late S phase [17].) Gemcitabine—radiation interactions are complex: reviewing pre-clinical data and clinical trials, it was noted [201] that 'the mechanism of radiosensitization by gemcitabine is still not fully elucidated, and the optimal treatment schedule still has to be defined'. The need for phase II trials of radiation with gemcitabine was stressed [8]. A succinct but comprehensive survey of the mechanistic basis for the use of both 5-FU and analogues, and gemcitabine, stressed the importance of inappropriate progression of cells into S phase [3].

A review of radiosensitizing nucleosides [196] included a summary of experience with hydroxyurea as a radiation sensitizer because its primary mechanism of cytotoxicity is related to the inhibition of ribonucleotide reductase. A number of clinical trials, with mixed results, were summarized. Hydroxyurea can also modulate both fluoropyrimidine- and IUdR-mediated radiosensitization.

Radiosensitizers that Influence the Nature or Repair of DNA Damage

Categorising radiosensitizers into well-defined types is particularly difficult when some chemicals have dual effects. Nitroimidazoles with sidechain alkylating functionality (e.g. RSU1069) are a simple example (see above). The use of redox metals with possible dual functionality discussed above (copper, gadolinium) is another; platinum a third. Cisplatin and carboplatin have been studied in some detail in combined treatments with radiation, with activity apparently as 'conventional' electron-affinic hypoxic cell radiosensitizers and as inhibiting post-irradiation repair [202,203]. There was a time-dependent increase in the extent of DNA double-strand breaks after irradiation of several cell lines *in vitro* with high concentrations of carboplatin [204]. Dual-function platinum/nitroimidazole complexes have also been evaluated [205], extended to non-platinum analogues [206,207]. A recent detailed discussion clearly set out the several mechanisms that might be involved in the interaction of platinum complexes with radiation [3]. Numerous clinical trials have been reported involving cisplatin or carboplatin and radiotherapy, often with additional cytotoxins and usually involving drug treatment with a few of the radiation fractions because of toxicity. An illustrative, recent review of the chemoradiation paradigm in the context of non-small cell

lung cancer [208] summarised the position as 'The use of single-agent cisplatin has already demonstrated major radiosensitizing effects whereas the radiosensitizing properties of concurrent application of the single agent carboplatin have not been observed in controlled trials'. Another review [209] focused on squamous cell carcinomas of the head and neck and oesophageal carcinomas, concluding with respect to the former site that concomitant chemo- and radiotherapy with platinum-based drugs does provide a small survival gain compared with radiation alone, and for the latter site that preoperative, cisplatin chemoradiotherapy modestly improves outcome over surgery alone, but with additional morbidity. Early trials involving radiation with a newer analogue, oxaliplatin, have been summarised [7].

β-Lapachone is a naturally occurring 1,2-naphthoquinone and as such might be considered an electron-affinic radiosensitizer. However, it was shown to inhibit topoisomerase I and radiosensitize human malignant melanoma cells *in vitro* when added *after* irradiation [210]. Other DNA topoisomerase I-targeted drugs have been described as radiation sensitizers effective when given before, but not after, radiation, including derivatives of camptothecin* [211]. Although the mechanism of topoisomerase I-mediated radiosensitization 'remains [in 2004] largely unknown' [211], a role for Ku86, important in the non-homologous end-joining pathway in DNA repair, has since been shown [212]. Topotecan is a derivative of camptothecin, which inhibits topoisomerase I in S phase cells; it has been suggested that it may be effective in the treatment of brain metastases by radiation [213]. AQ4 is an anthraquinone that is a DNA intercalator and topoisomerase II poison; the di-*N*-oxide prodrug, AQ4N*, is activated selectively in hypoxic cells, and when combined with radiation greatly enhances growth delay in murine tumours [214]. In related work, intratumour injection of an activating cytochrome CYP2B6 vector showed further enhancement over AQ4 N and radiation [215]. The agent is currently in phase I/II clinical trial in combination with radiotherapy and temozolomide in patients with glioblastoma multiforme. Temozolomide* is a prodrug for an alkylating agent that methylates guanine at O-6; clinical trials of temozolomide plus radiation in malignant glioma have recently been summarised [216]. In a large phase III European study, the 2-year progression-free survival was increased from ~2 to 11% by the inclusion of temozolomide with radiotherapy, and overall survival at 2 years from ~10 to 27%, 'one of the most significant improvements in survival found in any phase III clinical study in newly diagnosed GBM [glioblastoma multiforme] patients within the past several decades' [216].

PARP is a nuclear enzyme facilitating DNA base excision repair; the therapeutic potential of PARP inhibitors in a broad context has been reviewed [217]. Whereas the paper noted the early work with nicotinamide in this context, emphasising its low activity as a PARP inhibitor and thus reinforcing the assignment of effects on tumour oxygenation to other mechanisms (see above), a recent study described a much more potent PARP inhibitor that has the specificity and activity *in vivo* to enhance radiotherapy [218]. Treatment with AG14361* before irradiation significantly increased the sensitivity to radiation therapy of mice bearing LoVo xenografts: irradiation alone (2 Gy daily for 5 days) caused a 19-day tumour growth delay, extended to a 37-day delay in mice treated with AG14361 before irradiation.

The ataxia telangiectasia mutated (ATM) protein kinase plays a critical role in regulating cell cycle arrest and DNA repair, linked to dramatically enhanced radiation sensitivity, and is attracting attention as a target for radiosensitizers [219]. Inhibitors of the ATM kinase, such as wortmannin and caffeine, sensitize cells *in vitro*; the former is very reactive towards proteins non-specifically, including effects on enzymes catalysing non-homologous end-joining (an important DNA repair mechanism in normal cells), and the latter is active only at undesirably high concentrations (around 0.2 mM). Pentoxifylline is a drug related to caffeine that has also been shown to have vasoactive properties and to modify tumour oxygenation as described above [56,57]; studies of both caffeine and pentoxifylline in the context of ATM as a target have been reviewed [219]; neither appeared clinically useful, although a more recent overview [8] was more optimistic. However, there are some interesting points. Caffeine is more effective as a radiosensitizer in cells that lack normal p53 function, by a factor of 1.4—2.8-fold, and another methyl xanthine, lisofylline*, radiosensitizes cells lacking p53 at clinically tolerable concentrations [220].

Radiosensitizers Targeting Proteins Involved in Cell Signalling, and Growth Factors

This family of radiosensitizers is the newest to be investigated, although why they are often termed 'molecular agents' or the targets 'molecular' is unclear: misonidazole and DNA are molecules too. Three recent reviews focusing on this area illustrate the diversity of approaches [7,221,222]. Proteins involved in cell signalling and growth factor receptors are the main categories of target. In the former, the proteins most studied in the context of radiation therapy are probably the Ras family, which is of wider interest in cancer therapy because they control signalling pathways that are key regulators of cell growth and transformation [223]. There is substantial evidence linking Ras proteins to radioresistance [23,224]. The functionality of Ras is in turn linked to the addition of a farnesyl isoprenoid moiety, and farnesyl transferase inhibitors such as L-778,123* have been evaluated as radiosensitizers [225]. Pre-clinical studies showed that such inhibitors can reverse radioresistance in human tumour xenografts expressing the mutant *Ras* oncogene, and phase I/II clinical trials recently commenced. In one study, L-778,123 was used with concurrent radiotherapy to treat patients with locally advanced pancreatic cancer. The data confirmed pre-clinical observations that there was no increase in radiation-induced normal tissue damage, and

therefore the approach has the potential to increase the therapeutic index.

There is intense activity in the area of epidermal growth factor receptor (EGFR) inhibitors, which might mediate cell growth, differentiation and survival [3,8,226]. The potential mechanisms of radiosensitization by EGFR are complex [226]. Enhanced anti-tumour activity with vandetanib* (ZD6474) and radiation was reported in a pre-clinical study [227] using a model previously found to be unresponsive to one selective EGFR tyrosine kinase inhibitor. However, scheduling of ZD6474 relative to radiotherapy had a profound effect on the enhancement: sequential, chronic administration of ZD6474 after the radiation treatment significantly enhanced growth delay. In contrast, the response after concurrent treatment with ZD6474 and radiotherapy revealed no significant interaction between the two modalities. A positive phase II trial evaluating an anti-EGFR antibody, cetuximab, and radiotherapy, involving 424 patients with advanced head and neck cancer, has been reported [228]. At ~54 months, the median duration of overall survival was 49 months for combined therapy and ~29 months for radiotherapy alone, but an editorial expressed caveats [229]. Harari and Huang [230] reviewed the use of cetuximab and other EGFR inhibitors, including gefitinib* (Iressa), noting the highly promising pre-clinical data, but concluding that 'the overall clinical gains to date … are modest for the global cancer population'.

Cyclooxygenase (COX)-2 is often over-expressed in cancer and is linked to resistance to cytotoxic agents. The inhibition of COX-2 has been found to enhance the tumour response to radiation in pre-clinical studies, and COX-2 expression has been linked to tumour radioresistance [8]. An example being evaluated clinically in the radiotherapy of lung cancer is celecoxib* [231], which was shown in recent mechanistic studies [232] to down-regulate the expression of Ku70 protein and to inhibit the kinase activity of DNA-PKcs, important in repairing double-strand DNA breaks. It was also suggested that NFκB may play a role in mediating the effects of celecoxib [232].

Finally, other targets that are attracting attention in the context of radiation therapy include anti-angiogenesis inhibitors [233]; a sulphoglycolipid has been identified as a candidate radiosensitizer [234]. The molecular target is unknown, although the agents are inhibitors of DNA polymerases. The potential use of inhibitors of the hypoxia-inducible factor HIF-1 has been discussed [54]. Vascular endothelial growth factor and Rad51 enzymes (catalysing DNA double-strand break repair) are other new targets for radiosensitization, discussed in a recent review [200]. Membrane targeted drugs have been reviewed as putative radiosensitizers [235].

Suppression of Radioprotective Substances

Depletion of intracellular thiols is the obvious approach to take in this context [9]. Indeed, thiol-reactive chemicals were among the first radiosensitizers to be studied [236—239]. Some agents were both electronic-affinic and depleted thiols, and attention has been drawn in the above discussion (and in Table 1) to the artefacts that can arise in testing these chemicals *in vitro* because reaction binds or oxidises all the cellular thiols while leaving a large fraction of reagent unchanged. This gives rise to an elevated expectation of efficacy impossible to achieve *in vivo*. In addition to the nitroimidazoles where thiols displace halogen or other 'leaving groups' [172], powerful electron-withdrawing substituents, such as —CHO, can activate the nitro substituent in 2-nitroimidazoles to displacement by thiol, catalysed by glutathione-*S*-transferases [240].

A much more sophisticated and controllable approach to depleting intracellular thiols is the inhibition of steps in their biosynthesis: the application of L-*S*-buthionine sulphoximine* (BSO) was a major advance [241]. A significant enhancement in the radiosensitizing efficiency of misonidazole *in vitro* was seen on pre-incubation with BSO to deplete cellular glutathione (GSH) [242]. However, this was not generally translated to *in vivo* models to anything like the same extent, despite protocols showing good depletion of average tumour GSH levels [243—246]. An explanation might have been the poor diffusion of the rather polar BSO molecule to hypoxic cells distant from the vasculature; this seems not to be the case, although heterogeneity of GSH levels in tumours was indicated [247]. Microregional heterogeneity of GSH in cervical carcinoma, with higher GSH levels more susceptible to BSO-initiated depletion in hypoxic areas, has been reported [248,249].

BSO has been evaluated in several clinical trials, mostly in the context of chemotherapy regimens where GSH is protective, for example with melphalan [250,251], which have shown the clinical feasibility of depleting tumour GSH levels. There do not seem to have been recent studies involving BSO and radiotherapy, although there remains active interest in alternative approaches to deplete GSH in tissues [252], and new strategies to confer tumour selectivity would have obvious application in both radiotherapy and chemotherapy. It should be born in mind that GSH is not the only cellular thiol: levels of both cysteine [253,254] and protein thiols [255] must also be considered in this context. GSH is not the only antioxidant, either: ascorbate is highly reactive towards oxidizing DNA base radicals [256], and competitive radiation dose modification by ascorbate and misonidazole in thiol-depleted cells has been reported [257].

Conclusions

There are a wide variety of routes by which chemicals can interact in some way with radiation damage to offer potential therapeutic gain. The levels of early chemical damage such as DNA strand breaks can be enhanced, as with oxygen, 'electron-affinic' compounds and nitric oxide. The tumour microenvironment can be modified to reduce acute hypoxia, as with carbogen and nicotinamide. Radioresistant subpopulations of cells can be targeted, such as hypoxic cells with tirapazamine. The efficiency of DNA

repair can be inhibited, with the potential to exploit hypoxia to deliver the new generation of potent repair inhibitors (or other 'molecularly targeted' drug) selectively to tumours via the 'trigger—effector' concept, or as directly hypoxia-activated prodrugs. Tumour proliferation can be exploited by S phase-specific uptake of halopyrimidines, which can act as dissociative electron 'sinks' to enhance radical damage when incorporated into DNA or affect nucleoside and nucleotide metabolism. Enzymes involved in signal transduction pathways, cell cycle checkpoints, growth factors, etc. … can all be targeted.

To change the status of radiosensitizing chemicals into clinically useful drugs raises many questions. Coleman and Mitchell [258] summarised these (selecting and paraphrasing some key points or illustrations in brackets) as:

- 'What is the target of the modifier? [DNA, transcription factor, enzymes, receptor, signalling molecule …]
- Is the target stable? [cell cycle variation, heterogeneity, resistance …]
- Can the target be reached? [pharmacology, distribution (macro/microscopic) …]
- What is the optimal schedule? [pharmacokinetics, cell cycle perturbation …]
- Can the radiation modifier be used throughout a course of fractionated radiation therapy? [drug toxicity, every treatment or selected; if latter, when? …]
- Selectivity: tumor versus normal tissue? [is modifier plus radiation better than more radiation …]
- What is the design of the clinical trial?' [choice of end point, sufficiently large study …].

It is thus obvious that many skills have to be brought into play to be successful in this area. Reviewing in 1996 the treatment of cancer with radiation and drugs, Tannock [5] concluded: 'Clinical gains from combined treatment with radiation and drugs have been small. New, mechanistically based approaches to combined treatment are required'. In preparing the present overview, the author has been reminded that there has been little advance in the last two decades in understanding the mechanisms of how the even well-established chemical radiosensitizers actually work at the molecular level. Some newer radiosensitizers have reached clinical trials without the mechanistic basis for their putative action being properly understood.

The limitations of *in vitro* models should not be overlooked. The author is particularly concerned that ascorbate is present at high concentrations in mammalian tissues [259], yet normally completely absent in *in vitro* models used in radiobiology. Ascorbate is well known to radiation chemists as highly reactive towards some DNA radicals [256], as an important 'radical sink' in biology more generally [260], and as a potential pro-oxidant with redox metals [169]; as noted above, adding ascorbate diminishes radiosensitization by misonidazole in thiol-depleted cells *in vitro* [257]. As attention focuses on newer targets in cell biology, it is important not to overlook simpler molecules such as ascorbate and nitric oxide.

In conclusion, there are several new approaches in the field of chemical radiosensitizers that show promise, but their mechanistic basis is poorly researched. Some almost-forgotten radiosensitizing chemicals, such as nitric oxide, are presenting exciting new possibilities, and there is still life left in the 'old dog', oxygen. However, it is no use focusing on 21st-century cell biology while neglecting even 1950s' chemistry, or indeed seeking to test sophisticated chemistry using inappropriate biological models.

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