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Overview

Overcoming Radioresistance: Small Molecule Radiosensitisers and Hypoxia-activated Prodrugs

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Abstract

The role of hypoxia in radiation resistance is well established and many approaches to overcome hypoxia in tumours have been explored, with variable success. Two small molecule strategies for targeting hypoxia have dominated preclinical and clinical efforts. One approach has been the use of electron-affinic nitroheterocycles as oxygen-mimetic sensitisers. These agents are best exemplified by the 5-nitroimidazole nimorazole, which has limited use in conjunction with radiotherapy in head and neck squamous cell carcinoma. The second approach seeks to leverage tumour hypoxia as a tumour-specific address for hypoxia-activated prodrugs. These prodrugs are selectively activated by reductases under hypoxia to release cytotoxins, which in some instances may diffuse to kill surrounding oxic tumour tissue. A number of these hypoxia-activated prodrugs have been examined in clinical trial and the merits and shortcomings of recent examples are discussed. There has been an evolution from delivering DNA-interactive cytotoxins to molecularly targeted agents. Efforts to implement these strategies clinically continue today, but success has been elusive. Several issues have been identified that compromised these clinical campaigns. A failure to consider the extravascular transport and the micropharmacokinetic properties of the prodrugs has reduced efficacy. One key element for these 'targeted' approaches is the need to co-develop biomarkers to identify appropriate patients. Hypoxia-activated prodrugs require biomarkers for hypoxia, but also for appropriate activating reductases in tumours, as well as markers of intrinsic sensitivity to the released drug. The field is still evolving and changes in radiation delivery and the impact of immune-oncology will provide fertile ground for future innovation.

Key words: Hypoxia; hypoxia-activated prodrugs; radiosensitisers; radiotherapy

Statement of Search Strategies Used and Sources of Information

This overview was based on a sunrise educational presentation on the topic at the 62nd Annual Radiation Research Meeting, 2016 and has been updated to reflect recent developments in the field.

Introduction

Tumour hypoxia is a well-validated target for oncology drug development, particularly in the context of radiotherapy. Five decades of drug discovery have led to few tangible clinical successes. The failure to capitalise on this opportunity in the era of 'targeted therapy' is an obvious anomaly. In this overview we examine the argument for targeting hypoxic tumour cells and explore recent efforts to develop hypoxic cell sensitisers and hypoxia-activated prodrugs (HAPs). We aim to give insight into the challenges and opportunities presented by this ubiquitous target.

Hypoxia as a Drug Target

Hypoxia is an important element of the tumour microenvironment and plays an active role in tumour progression and response to treatment [1,2]. Oxygen supply is diffusion-limited in small, avascular tumours and is also compromised by disordered and dysfunctional tumour vasculature in larger tumours [2,3]. This oxygen deficiency is

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exacerbated by increased metabolic demand within tumours, resulting in pathophysiological hypoxia. Identification of the molecular signalling pathways mediated by hypoxia-inducible factors revealed the pernicious influence of hypoxia [4,5]. Hypoxia plays a role in the suppression of cell death [6,7], and immune responses [8–10], in the initiation of angiogenesis [11] and vasculogenesis [12], in the activation of a glycolytic shift in metabolism [13,14], and acts to enhance invasion [15] and metastasis [16–18]. Hypoxia also promotes genomic instability through increased production of reactive oxygen species [19] and suppression of DNA repair processes [20,21]. The significance of hypoxia in resistance to cytotoxic therapy has driven extensive efforts to modify hypoxia for clinical benefit [22–26].

Tumour hypoxia is a moving target, both spatially and temporally, and it is a continuum, with oxygen concentrations varying from about 2–3 μM, where hypoxia-inducible factors are activated, through to radiobiological hypoxia at about 0.1 μM and below [27]. Consequently, hypoxia presents a challenging prescription for drug design. Diffusion-limited hypoxia exists at some distance (up to 200 μm) from functional vessels and any potential drug has to diffuse to these regions [28]. This demand requires balancing lipophilicity with aqueous solubility and metabolic stability with reactivity, collectively described as extravascular transport (EVT). These issues were not fully considered in early drug approaches and poor EVT can compromise efficacy [29–32].

Early efforts focussed on hypoxia modification: fractionated radiation [33], hyperbaric oxygen [34] and carbogen breathing [35]. A range of small molecule approaches to radiosensitisation were also explored, including suppression of intracellular thiols, radiation-activated cytotoxins, halogenated pyrimidine sensitisers, repair inhibitors and oxygen-mimetic radiosensitisers [23]. Overall, hypoxic modification provided chequered results [36]; limited by poor trial design and lack of target selection markers for patients with hypoxic tumours. Although many individual trial results failed to provide benefit, when taken together these interventions were shown to provide improved outcomes [26]. That tumour hypoxia could be turned to advantage evolved from the observation of selective toxicity to hypoxic cells by nitroimidazole radiosensitisers such as misonidazole and work on the reductive activation of mitomycin C. As the mechanistic differences between radiosensitisation and hypoxia-selective cytotoxicity emerged, the concept of hypoxia as a tumour-selective drug target was soon in vogue [37]. Principally DNA-interactive cytotoxins, these HAPs were activated by one-electron reductases in the absence of oxygen to selectively kill hypoxic cells. The concept of hypoxia as a tumour-selective address has been further elaborated with the use of HAPs to release molecularly targeted agents.

Radiosensitisers

Hypoxic cells contribute to resistance in radiotherapy [26,38–41]. Hypoxic cells are two-to three-fold less sensitive to radiation because oxygen is required to convert radiation-induced DNA radicals to strand breaks (Figure 1) [42]. The development of oxygen-mimetic nitroimidazole radiosensitisers was a direct effort to overcome this resistance. They consist of an electron-affinic nitroheterocycle and a sidechain that modulates the physicochemical and pharmacokinetic properties [43–45] (Figure 2).

Metronidazole was the prototypic radiosensitiser and is a weakly electron-affinic 5-nitroimidazole. It was first administered, as a hypoxic cell sensitiser, in 1973 to patients requiring large doses (4–10 g) to achieve responses [46]. An unconventional radiation dose-fractionation regimen was used to overcome the dose-limiting gastrointestinal toxic effects of metronidazole [47]. Extensive studies at the Gray Laboratory led to the identification of the more potent, electron-affinic misonidazole [43]. Misonidazole showed promising radiosensitisation in a range of tumour models. However, delayed peripheral neuropathy limited its efficacy [46,48–52]. A variety of treatment regimens were explored to maximise sensitisation while limiting neurotoxicity, thus making interpretation of results challenging [26,46].

Designing more polar analogues with increased systemic clearance to minimise neurotoxicity was only partially successful with the identification of etanidazole [53–55]. Etanidazole displayed excellent activity in tumour models [53], but failed to provide benefit in head and neck cancer [56,57] and in prostate cancer [58]. The more polar doranidazole underwent clinical investigation in non-small cell lung cancer (NSCLC) [59] and pancreatic cancer in conjunction with intraoperative radiotherapy [60] and displayed a small survival advantage [61].

The 5-nitroimidazole nimorazole is a relatively weak sensitiser [44,62,63], but is well tolerated [64–67]. Nimorazole is active in combination with fractionated

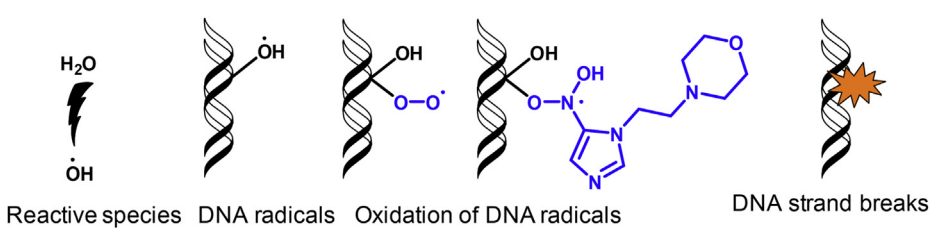

Fig 1. Radiation generates reactive species, e.g. hydroxyl radical (•OH) from water. The reactive species reacts with DNA to produce DNA radicals. In the presence of oxygen, or an oxygen-mimetic radiosensitiser (e.g. nimorazole), oxidation of the DNA radicals occurs and eventually results in DNA strand breaks.

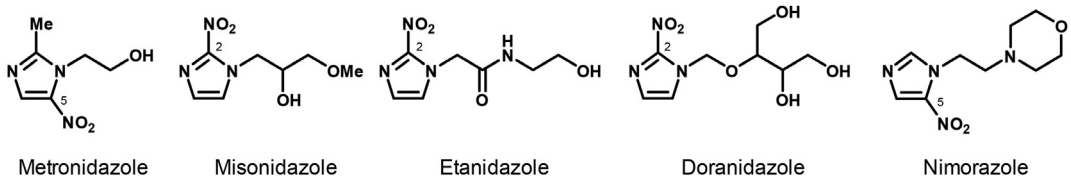

Fig 2. Selected nitroimidazole radiosensitisers.

radiotherapy for head and neck squamous cell carcinoma (HNSCC) [68], but is in use only in Denmark [69]. A retrospective analysis of patients with HNSCC undergoing fractionated radiotherapy used a hypoxic gene signature [70] to identify patients with hypoxic tumours. This study showed that nimorazole conferred a significant benefit, but only to patients with hypoxic tumours [71,72]. Nimorazole has been evaluated in conjunction with accelerated radiotherapy, but the trial was closed early due to low patient recruitment [67]. The results suggested treatment benefit compared with radiotherapy alone, particularly in the subset of patients with more hypoxic tumours [67]. When combined with accelerated radiotherapy and weekly cisplatin, nimorazole was well tolerated [73] and is currently undergoing evaluation in conjunction with prospective use of the hypoxic gene signature to stratify patients (NCT01880359). Nimorazole is being evaluated in combination with intensity-modulated radiotherapy in patients unsuitable for concomitant chemotherapy or cetuximab (NIMRAD, NCT01950689) [74].

Several factors have contributed to the limited success of radiosensitisers. Their use with fractionated radiotherapy, where fractionation of the radiation dose is designed to allow tumour reoxygenation between fractions, reduces the potential for radiosensitisation [33,75,76]. This requires administration of radiosensitiser with each fraction of radiation; a schedule that was unachievable for misonidazole and etanidazole due to cumulative peripheral neurotoxicity [77,78]. Most importantly, the clinical trials were conducted without prospectively identifying patients with hypoxic tumours, despite considerable heterogeneity in the level and extent of tumour hypoxia between patients [79]. In all fairness, many of these trials were conducted before the advent of 'targeted therapy' and the demonstration of the clinical (and commercial) benefit in limiting patient selection to those with the target. The new clinical trials to validate prospective use of this hypoxic gene signature with nimorazole will hopefully encourage others to co-develop hypoxia biomarkers.

Hypoxia-activated Prodrugs

The demands of drug delivery to target tumour hypoxia invoke a prodrug approach, where an inert prodrug diffuses through the extravascular tissue to hypoxic regions before being activated through a bioreductive 'trigger' linked to an 'effector' [80,81]. HAPs may be grouped into five chemical classes: nitroimidazoles, nitrobenzenes, quinones, N-oxides and metal complexes and these have been reviewed extensively [22,24,25,81–86]. Our focus is on HAPs explored in recent clinical studies and the lessons derived from these studies.

The common mechanistic element across these classes is the one-electron reduction of the bioreductive trigger by an obligate one-electron reductase to form a radical anion (Figure 3). In the presence of oxygen, this radical anion is reoxidised to the trigger with concomitant formation of superoxide, which is readily processed by protective mechanisms [87]. Under hypoxia, the radical anion undergoes further reduction to an active drug that mediates cellular toxicity, fragments or rearranges to produce a reactive species or activated drug (effector). A major shortcoming in this mechanism is that obligate two-electron reductases may activate the prodrug in an oxygen-insensitive manner, undermining tumour selectivity. The kinetic balance between the back reaction of the radical anion with oxygen and the forward reactions (Path a or b, Figure 3) provides the basis of hypoxic selectivity. This balance is influenced by the intrinsic sensitivity of each radical anion to oxygen with the K_{O2} (the K_i for inhibition by oxygen). The K_{O2} for nitroimidazole (e.g. evofosfamide) and nitrobenzene (e.g. PR-104) radical anions are relatively low at about 0.1 μM O_2 [88]. By contrast, aromatic N-oxides (e.g. tirapazamine; TPZ) have a higher K_{O2} of about 3 μM [89]. These differences place distinct demands on prodrug behaviour.

Mitomycin C was the earliest example of a HAP [90,91] where sequential one-electron reduction by oxidoreductases could lead to cytotoxic species (Path a, Figure 3)

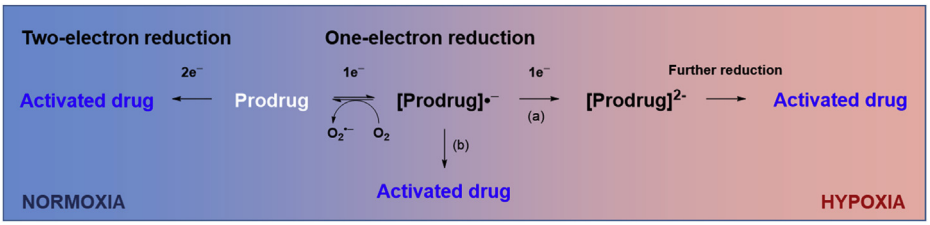

Fig 3. Generalised scheme showing activation of hypoxia-activated prodrugs. Prodrugs are metabolised by one-electron reductases to a radical anion, which may be reoxidised by oxygen. In the absence of oxygen, the radical anion may: (a) be further reduced to an active drug or (b) fragment or rearrange directly to an active drug. Two-electron reductases may also reduce the prodrug in an oxygen-insensitive manner.

[92,93]. However, oxygen-insensitive two-electron reduction compromises hypoxic selectivity [94]. Other quinones, such as porfiromycin [95] and apazaquinone [96,97], were explored, but provided no clear advantage [97,98].

TPZ (Figure 4) undergoes one-electron reduction to a radical anion, which is protonated to give an intermediate TPZ radical [99,100], subsequently rearranging to an oxidising radical (Path b, Figure 3). Despite considerable debate as to the identity of this species: either hydroxyl radical [101,102] or benzotriazinyl radical [103–105], the resulting radical interacts with DNA to form a complex spectrum of damage, including double-strand breaks (DSB) and clusters of single-strand breaks [106–109]. Two-electron reduction leads to the 1-oxide and then the nor-oxide, neither of which significantly contribute to cytotoxicity. The oxidising radicals are short lived and do not show a bystander effect, where activated effectors diffuse to adjacent cells [110,111]. This limitation is offset by a relatively high K_{O2} allowing TPZ to kill tumour cells at intermediate hypoxia [89] that may be most influential in repopulating the tumour after radiotherapy [112]. TPZ showed significant hypoxic selectivity *in vitro* [88,113,114] and *in vivo* in combination with radiation [115,116] and cisplatin [117].

TPZ displayed activity in advanced NSCLC, in combination with cisplatin, providing significant increases in response rates and overall survival compared with cisplatin alone [118], but was not superior to etoposide combined with cisplatin [119]. TPZ was also explored in cervical cancer [120–124]. However, the most informative studies came from the combination of TPZ and chemoradiotherapy in HNSCC [125,126]. A phase II study was conducted with ^{18}F-misonidazole positron emission tomography (PET) imaging for hypoxia. TPZ, in combination with cisplatin chemoradiotherapy, showed a trend for increased locoregional control and failure-free survival over 5-fluorouracil plus chemoradiotherapy, but only in patients with hypoxic tumours [125,127,128]. The hypoxia biomarker was not adopted in the subsequent randomised phase III trial, where TPZ did not significantly improve overall survival or local tumour control [126]. Retrospective analyses identified poor radiotherapy quality control, a trend in favour of TPZ for time to locoregional failure in treatment compliant patients [129] and the impact of HPV p16 status [130]. However, these observations were insufficient to salvage the future of TPZ and clinical development was halted. Biomarkers for patient selection were ignored with predictable results. Unfortunately, this clinical failure also reduced interest in second-generation TPZ analogues, such as SN30000, where optimisation of EVT [32] and micropharmacokinetic properties [131] led to superior activity in preclinical studies [109,132–134].

The dinitrobenzamide mustard PR-104 was identified from a medicinal chemistry programme [135–138] based on the initial identification of nitroreduction as an electronic switch for the activation of nitrogen mustards [139–141]. PR-104 is a phosphate pre-prodrug that is hydrolysed rapidly by phosphatases, releasing the PR-104 alcohol. This is reduced in a hypoxia-selective manner by one-electron reductases such as P450 oxidoreductase [142] and other diflavin reductases [143]. The eventual hydroxylamine and amine products activate the mixed bromomesylate mustard to generate DNA cross-links (Path a, Figure 3) [144]. PR-104 activation occurs at relatively low oxygen tensions and this mandates a bystander effect for effector to diffuse and induce cell death in adjacent cells at intermediate hypoxia [111,145]. PR-104 showed single-agent activity and synergistic activity in combination with radiation and chemotherapy in multiple xenograft models [146]. Single-agent activity suggested unexpected oxygen-insensitive activation [147,148] and the aldo-keto reductase isoform 1C3 (AKR1C3) was identified as the two-electron reductase responsible [147].

Phase I clinical trials showed dose-limiting haematological toxicity [149–151], with AKR1C3 expression in myeloid progenitor cells suggested as a contributor to this toxicity [152]. An alternative strategy targeting cancers with high AKR1C3 expression [153–155] was suggested, but as AKR1C3 is not the sole determinant of PR-104 activity, a better suite of predictive biomarkers is needed. Although PR-104 displayed strong activity in combination with radiotherapy [146], it was developed in combination with chemotherapy (docetaxel, sorafenib) [156]. Significantly, it was not developed with a biomarker for hypoxia.

A new nitrobenzamide mustard analogue CP-506, based on PR-104, but without AKR1C3 sensitivity, has been reported in brief by Convert Pharmaceuticals [157,158]. CP-506 displays hypoxia-selective killing of triple-negative breast cancer, lung and pancreatic tumour cells *in vitro* and *in vivo* and also displays a bystander effect. Further details are awaited with interest.

Threshold Pharmaceuticals extended initial studies of nitroheterocyclic prodrugs of phosphoramidate mustards [159,160] to identify evofosfamide (TH-302), a nitroimidazole prodrug of bromoisophosphoramide mustard, as a HAP [161]. Reduction of the 2-nitroimidazole trigger unit initiates fragmentation and release of the phosphoramide

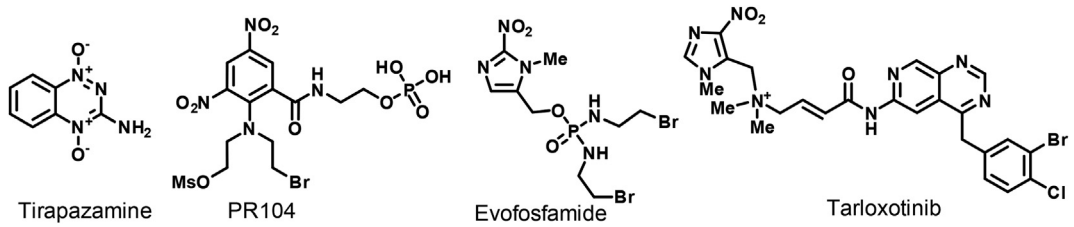

Fig 4. Selected hypoxia-activated prodrugs.

mustard (Path b, Figure 3) [162], but the released effector does not display a bystander effect [163]. Evofosfamide displays striking hypoxic selectivity *in vitro* [161,162,164] and impressive preclinical activity as a single agent [165] or in combination studies in a variety of tumour xenograft models [162,166]. Evofosfamide was the subject of a number of clinical studies, with positive results from two phase II clinical studies in combination with doxorubicin in soft sarcoma patients [167] and gemcitabine in pancreatic cancer patients [168]. These results were not replicated in phase III registration trials (NCT01746979 and NCT01440088) [169] and most studies have been terminated. The development of the PET imaging agent, ^{18}F-HX4, as a biomarker for hypoxia [170,171], and its application with evofosfamide in conjunction with radiotherapy [172], lagged behind the development of evofosfamide with chemotherapy. Despite evidence for activity in combination with radiation [172,173], there was only one (discontinued) clinical trial in combination with radiotherapy (NCT02598687).

Hypoxia-activated Prodrugs of Molecularly Targeted Agents

The focus on tumour-selective delivery by HAPs has recently shifted to include delivery of molecularly targeted agents for which normal tissue toxicity may be limiting, especially within the radiation field. Trials assessing the radiosensitisation of epidermal growth factor receptor (EGFR) small molecule inhibitors have yielded promising response data across a range of cancer types. However, their use has also been associated with substantial toxicity [174,175].

Tarloxotinib, a 4-nitroimidazole quaternary ammonium salt of an irreversible EGFR/HER2 inhibitor, was designed to improve the therapeutic index of EGFR inhibitors through tumour-selective drug delivery. Tarloxotinib is metabolised under hypoxic conditions to release a potent EGFR inhibitor, resulting in hypoxia-selective antiproliferative activity (Path b, Figure 3). Tarloxotinib was more active than cetuximab in FaDu HNSCC xenografts and was more active than afatinib in A431 skin squamous cell carcinoma (SSCC) xenografts, at clinically relevant doses [176]. Two proof-of-concept phase II trials were opened by Threshold Pharmaceuticals, recruiting patients with EGFR-mutant, T790M-negative NSCLC [177] and HNSCC and SSCC [178]. Importantly, baseline measurement of hypoxia using ^{18}F-HX4 PET imaging was included in both studies and biomarkers for intrinsic sensitivity were under examination [179]. However, discontinuation of both trials due to a failure to meet interim milestones was announced in 2016 [180], but no results have been formally published.

Prodrugs of DNA Damage Response Inhibitors

Radiotherapy produces DNA DSB as the principal cytotoxic lesions. All cells have highly organised DNA damage response (DDR) mechanisms to repair DNA damage. Two principal repair mechanisms deal with DNA DSBs: homologous recombination repair uses a sister chromatid to effect high-fidelity repair in S and G2 phases of the cell cycle, whereas non-homologous end-joining (NHEJ) results in error-prone rejoining of chromosomes throughout the cell cycle. These repair mechanisms also engender resistance to cytotoxic chemotherapy. Loss of function in a particular DDR pathway may sensitise cancer cells to a particular cytotoxic drug through the persistence of the DNA lesions [181]. Therapeutic targeting of the DDR to augment the activity of cytotoxic chemotherapy and radiotherapy, and to overcome resistance, has an extensive history [182–184]. However, targeting the DDR therapeutically in the absence of a synthetic lethality situation [185,186] may enhance normal tissue toxicity as well as tumour cell toxicity.

NHEJ has been implicated as the main DSB repair pathway for radiation-induced damage, as inactivation of components of NHEJ has been shown to result in radiosensitive phenotypes [187–189]. Central to the function of NHEJ in recognising and repairing DNA DSBs is the DNA–protein kinase (DNA-PK) complex. DNA-PK inhibitors have shown strong radiosensitisation, both in cancer cells [190–193] and in xenograft models [190,191,194]. Two DNA-PK inhibitors (VX984 aka M9831; M3814 aka nedisertib) are currently in early phase trials, with nedisertib in combination with radiotherapy (NCT02516813). However, the extreme radiosensitivity of SCID mice with inactivating mutations in the DNA-PKcs gene *prkdc* [195] shows the inevitability that DNA-PK inhibitors will potentiate normal tissue toxicity within the radiation field [196]. Recent data have shown that although the combination of nedisertib with radiotherapy provided improvement in local tumour control in several human tumour xenograft models (FaDu, MiaPaCa, A549), it also markedly increased the incidence of radiation-induced skin toxicity [197].

Several other key targets within the DDR have been the focus of considerable activity for the development of radiosensitisers. Inhibition of ataxia-telangiectasia mutated (ATM) kinase radiosensitises tumour cells [198–200] and the ATM inhibitor AZ1390 is being advanced for the treatment of gliomas and brain metastases [201]. One report noted that mice bearing orthotopic glioblastoma tumours treated with AZ1390 and whole-head irradiation experienced mucositis, but that treatment with conformal radiotherapy and AZD1390 did not induce any overt toxicity [202]. This suggests that normal tissues within the radiation field may be sensitised by ATM inhibition.

The potential for HAPs to provide tumour-selective delivery of inhibitors of the DDR, and so improve their therapeutic index in combination with radiotherapy, is still at an early stage. A prototype HAP of a DNA-PK inhibitor was shown to sensitise hypoxic NCI–H460 cells to radiation [203] and a new analogue showed effective radiosensitisation of hypoxic SiHa tumour cells *in vivo* [204]. Similarly, a proof-of-concept HAP of checkpoint kinase 1 showed hypoxia-selective activation and cytotoxicity [205].

Conclusion and Perspectives

Given that radiosensitisers and HAPs are targeted therapies and that considerable tumour heterogeneity exists for hypoxia [79], it is critical for their clinical development to identify this target prospectively in patients. HAPs pose an additional challenge; requiring biomarkers for not only hypoxia, but also the activating reductases for the prodrug and intrinsic sensitivity to the effector. Although a plethora of biomarkers for hypoxia have been explored [206], a lack of consensus has hindered the clinical development of radiosensitisers and HAPs. Recent working group guidelines on future clinical research on radiosensitisers have highlighted the importance of biomarkers [207–209]. The failure to use a biomarker for hypoxia in the phase III trials of TPZ contributed to its clinical failure. By contrast, the use of a hypoxic gene signature, even retrospectively, was critical in the success of nimorazole in HNSCC.

Changes in radiation fractionation, such as stereotactic body radiotherapy, where there is less opportunity for reoxygenation [210,211], may provide new opportunities for new oxygen-mimetic radiosensitisers [212].

Clearly, the use of immune checkpoint inhibitors is transforming cancer therapy including radiotherapy [213]. Initial observations of radiotherapy-stimulated immune responses [214–216] are now being consolidated, with the combination of anti-CTLA-4 agents [217–220] or anti-PD-L1 agents [219,221] with radiotherapy. Numerous trials exploring the use of anti-CTLA-4 and anti-PD-1/PD-L1 agents in HNSCC are ongoing and the recent US accelerated approval of pembrolizumab in platinum-refractory recurrent/metastatic HNSCC illustrates the changing clinical landscape [222]. However, subpopulations of cells will remain resistant to this combined therapy for a variety of factors, including hypoxia and non-redundant checkpoint mechanisms [10,218]. It is clear that the addition of agents that eliminate hypoxic cells can be complementary with T-cell checkpoint blockade [223].

Tumour hypoxia remains a well-validated oncology target, with new agents under development and an evolution in strategy, moving from prodrugs of cytotoxins to molecularly targeted agents. Clear lessons from past campaigns have highlighted the need to address tumour micropharmacokinetics and EVT for prodrugs and effectors. It is critically important to use biomarkers not only for hypoxia, but also for the activating reductases and for intrinsic sensitivity to the release effector. The changing landscape of radiotherapy and (radio) immuno-oncology provides new opportunities for hypoxia targeting.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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