Exploring hypoxic biology to improve radiotherapy outcomes

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Abstract

Ionising radiotherapy is a well-established, effective cancer treatment modality, whose efficacy has improved with the application of newer technological modalities. However, patient outcomes are governed and potentially limited by aspects of tumour biology that are associated with radioresistance. Patients also still endure treatment associated toxicities owed to the action of ionising radiation in normoxic tissue adjacent to the tumour mass. Tumour hypoxia is recognised as key component of the tumour microenvironment and is well established as leading to therapy resistance and poor prognosis.

In this review, we outline the current understanding of hypoxia-mediated radiotherapy resistance, before exploring targeting tumour hypoxia for radiotherapy sensitisation to improve treatment outcomes and increase the therapeutic window. This includes increasing oxygen availability in solid tumours, the use of hypoxia activated pro-drugs (HAPs), targeting of hypoxia-regulated or associated signalling pathways, as well as the use of high-LET radiotherapy modalities. Ultimately, targeting hypoxic radiobiology combined with precise radiotherapy delivery modalities and modelling should be associated with improvement to patient outcomes.

Keywords: hypoxia, radiotherapy, radioresistance, targeted therapy, HIF, HAPs, DNA damage response

1. Introduction:

Ionising radiation is a type of high-energy electromagnetic wave that releases electrons from atoms and molecules generating highly reactive free radicals which can damage genomic DNA and result in cell death (1). Radiotherapy is one of the primary therapeutic strategies for many cancer types, either alone or in combination with surgery, chemotherapy, targeted therapy, and/or immunotherapy (2). For example, the standard treatment of nasopharyngeal carcinoma is radiotherapy, and early-stage laryngeal cancer patients are treated with radiotherapy as a primary therapy, with advanced laryngeal cancers also sensitive to chemoradiation (CRT) (3,4). More recently, radiotherapy has been explored with nwerer cancer treatment modalities, such as with immunotherapeutic agent pembrolizumab, which significantly increased responses in patients with metastatic non-small-cell lung cancer (5).

Unfortunately, treatment resistance leads to poor outcomes for some patients. A key aspect of tumour biology that affects ionising radiotherapy efficacy is the tumour microenvironment, in particular tumour hypoxia, as the cellular responses to ionising radiation are dependent on how well oxygenated a tissue is (6). In fact, 3-fold higher radiation doses are required in hypoxic conditions to achieve the same impact as in normoxic conditions, a factor noted as the oxygen enhancement ratio (OER) (7). Elevated hypoxic content in tumours has therefore been shown to be a factor of poor prognosis and therapy resistance in many tumour types (8-10). The oxygen levels at which significant radioresistance is observed (<0.13% O_2) are also known as radiobiological hypoxia (11). Hypoxia is therefore considered a significant challenge to ionising radiotherapy efficiency, so there is an expanding field of study looking at exploring strategies to radiosensitise hypoxic cells. This involves strategies such as increasing oxygen availability, hypoxia-activated pro-drugs (HAPs), or targeted therapies for hypoxia-regulated signalling. Interestingly, high-LET (linear energy transfer) radiotherapy modalities have been shown to be less dependent on oxygen levels than low-LET ionising radiation (7,12,13).

The aim of this review is to discuss how hypoxic biology impacts radiotherapy response, how hypoxic radiobiology can be explored therapeutically to avoid radiotherapy resistance, and

how high-LET modalities might be an alternative approach to hypoxia-induced ionising radiation resistance.

2. Hypoxia-mediated radiotherapy resistance

2.1. An overview of tumour hypoxia

In normal tissue the oxygen supply matches the metabolic requirements of the cells, whereas in tumour tissue oxygen consumption increases significantly and exceeds the supply, resulting in a drop of normal oxygen levels (pO_2) from about 20-80 mmHg to hypoxic levels < 5 mmHg, or even levels which can cause increased radioresistance (<1-10 mmHg or 0.13-1.3% O_2) (14). In particular, oxygen tensions of lower than 1 mmHg (<0.13% O_2) are associated with significant radiotherapy resistance and are therefore called radiobiological hypoxia (11).

Chronic hypoxia is caused by the long-term oxygen depletion, which can be derived from increased distance from blood vessels to the tissue, as well as permanent limitations in oxygen diffusion (15). Acute hypoxia occurs when a temporary disruption of blood flow to the tumour mass occurs because of the severely abnormal changes in the structure and function of tumour vasculatures, producing oxygen fluctuation in the tumour microenvironment (16). Because of this, solid tumours contain regions of cycling, or intermittent, hypoxia. The levels of hypoxia and proportion of the tumour that is hypoxic vary significantly due to the disorganized vessels with intermittent blood flow, which generate cyclic changes of oxygen concentrations, resulting in a dynamic microenvironment between hypoxic and reoxygenated states (17).

Hypoxic adaptation is underpinned by dramatic changes in gene expression patterns, and these are primarily regulated by the hypoxia inducible factors (HIFs) (18). HIFs can transactivate the expression of genes involved in key tumour promoting hallmarks, such as tumour angiogenesis, energy metabolism adaptation, cell death and autophagy, cell cycle regulation, metastatic spread (including the epithelial-mesenchymal transition, EMT), and both chemo- and radio-therapy resistance (19) (**Figure 1**). HIF consists of an oxygen-sensitive α subunit (HIF-α, which includes three isoforms: HIF-1α, HIF-2α and HIF-3α), and a constitutively expressed β subunit (HIF1-β). Under normoxic conditions, HIF-α is

hydroxylated by both PHDs (prolyl hydroxylases) and FIH (factor inhibiting HIF) (20). Proline hydroxylation within HIF-α's oxygen-dependent degradation domain (ODD) by PHDs allows HIF-α to be recognised and bound by the von Hippel-Lindau (VHL) E3 ligase, resulting poly-ubiquitination and subsequent degradation by the proteasome (21). However, under hypoxic conditions the PHDs are inhibited due to the lack of oxygen as a co-factor, leading to the rapid stabilisation of HIF-α protein levels and increased interaction with its co-activators p300 and CBP (CREB binding protein) (22). HIF-α then heterodimerises with HIF1-β, and the heterodimeric transcription factor then binds to hypoxia response elements (HREs) located in target gene promoters and transactivates these targeted genes (**Figure 1**) (23).

2.2. Hypoxia-mediated radiotherapy resistance

There are primarily two aspects by which hypoxia leads to radiotherapy resistance based on the mechanism of action of ionising radiation. As stated by the oxygen fixation hypothesis, during treatment with ionising radiation DNA radicals are formed either by direct ionisation or indirectly by interaction with free radicals generated by water radiolysis (24). Molecular oxygen rapidly interacts with these indirect radiation-induced DNA radicals leading to the production of single strand breaks and oxidized bases, which can be resolved into lethal double strand breaks (DSBs), leading to cell death (25). Therefore, in the absence of sufficient oxygen this process is inhibited, and the amount of DNA damage produced by radiation and its impact on cell viability is reduced. Other mechanisms by which hypoxic biology decreases ionising radiation efficacy include changes in ROS (reactive oxygen species) levels, inflammation signalling, and HIF-regulated signalling such as induction of angiogenesis and other tumour promoting pathways (**Figure 1**) (26). HIF-1α and HIF-2α expression have been shown to have poor prognostic value for response to radiotherapy or CRT (27,28). Counterintuitively, HIF-1α levels have been shown to increase after ionising radiation treatment through a variety of molecular mechanisms (29). Importantly, hypoxia can also drive increased genomic instability phenotypes through the clonal loss of tumour suppressor p53, repression of the expression of other tumour suppressive factors such as E2F1 as well as key players of DNA repair pathways such as double strand break repair (homologous recombination) and mismatch repair, such as RAD51, BRCA1, MLH1, amongst others (30-32). It is important to note that these latter resistance mechanisms are characteristic of, but not exclusive to, radiobiological hypoxia and are associated with

activation of DNA damage response signalling and DNA replication downregulation through decreased nucleotide signalling (11,33-35).

3. Increasing sensitisation to ionising radiation via increased oxygen availability

There are several approaches to target hypoxia-mediated radioresistance, and one of the longest established one is the direct or indirect modulation of oxygen levels in the tumour tissue to reduce hypoxic content and increase radiosensitisation. These utilise three main broad approaches: increasing oxygen diffusion to the tissue, reducing oxygen consumption, or using oxygen-mimetic molecules.

3.1 Increased oxygen diffusion

Hyperbaric oxygen (HBO) therapy has been used as a treatment for late radiation tissue injury by increasing the availability of oxygen in plasma, which improves oxygen tissue availability (36). A meta-analysis of several clinical trials to investigate the effect of hyperbaric oxygen as radiosensitisers in patients with squamous cell carcinoma of head and neck showed a significant improvement in overall radiation treatment response, as well as metastasis reduction (37). Radiotherapy after HBO breathing was found to be radiosensitised in a study using experimental models (38). However, this technique is not cost effective for broad clinical use in later study (37).

A phase two clinical trial investigated the effect of the combination of nicotinamide and carbogen (CON) on radiotherapy outcome for patients with advanced bladder carcinoma (39). Nicotinamide is a vitamin modified to enhance blood flow in the tumours and administered two hours before radiotherapy while carbogen refers to a gaseous mixture of 2% carbon dioxide and 98% oxygen inhalant (40). This study demonstrated improvement in overall response of 50% for those administered with the CON combination therapy, whilst radiotherapy alone only had a 38% overall response (39). A report from a phase III trial for laryngeal cancer also reported positive outcome of accelerated radiotherapy combined with carbon inhalation and nicotinamide compared to radiotherapy alone with a 93% control rate seen in patients with hypoxic tumours treated with the combination therapy (41).

Other approaches that enhance oxygen diffusion for reversing tumour hypoxia and improve radiotherapy are also under investigation. Trans sodium crocetinate (TSC) causes physical changes in blood plasma which results in rapid oxygen diffusion from the cell wall to the vascular wall (42). TSC was combined with temozolomide and radiotherapy on glioma cells and MRI imaging obtained before and after treatment showed a significant reduction in tumour size when compared with those treated with temozolomide and radiotherapy alone (43). TSC is being developed as a radiosensitiser for improving radiotherapy outcome in glioblastoma multiforme (GBM), pancreatic cancer, and brain metastases after a successful phase II clinical trial was completed (42).

Oxygen transport agents are also being explored to meet the challenges of hypoxia to radiotherapy. Preclinical investigation of liposome-encapsulated haemoglobin were shown to effectively reverse hypoxia in tumours (44). Specifically, the results showed a remarkable reduction of HIF-1α and improved radiation therapy outcome, as tumour growth was significantly inhibited (44). OMX is a recent oxygen carrier developed to target hypoxia and improve radiotherapy (45). Preclinical studies showed OMX reduced hypoxia significantly, enhancing T cell localization, and increasing CD8 accumulation and other cytotoxic activity previously impaired by tumour hypoxia (45). Fluorocarbon-based agents, through their gas-dissolving and chemically inert proprieties, can carry and diffuse oxygen at high concentrations (46). A phase II clinical trial (NCT03862430) in GBM, evaluating the combination of radiotherapy with NVX-108, a dodecafluoropentane-based perfluorocarbon (PFC) emulsion, is currently recruiting (47).

3.2 Decreased oxygen consumption

As well as increased oxygen delivery, suppressors of oxygen consumption have also been explored as radiosensitiser agents.

Nitric oxide (NO) is a free radical that plays a vital role as a vasodilator, as well as inhibitor of tissue oxygen consumption (48). The mechanism of NO in radiosensitisation is similar to those of oxygen-induced oxidative stress by stabilising radiation-induced DNA damage via the nitrosative stress pathways (49). The radiosensitising effect of NO has been shown both *in vitro* and in patients, including a phase II study indicating that NO can palliate hypoxia-induced progression in prostate cancer (50).

More recently, the anti-microbial agent atovaquone was found to rapidly decrease hypoxic content of tumours, and was identified as a suppressor of oxygen consumption through a high-throughput analysis of FDA-approved drugs (51). One clinical study found that atovaquone can increase tumour oxygenation and suppress hypoxic gene expression, therefore improve treatment outcomes for NSCLC patients (52).

Finally, papaverine, another FDA-approved agent, has also been shown as an ideal agent for radiosensitisation of hypoxic tumours as it reduces mitochondrial oxygen consumption (53). This anti-spasmodic drug was shown to increase oxygenation in tumour and enhanced radiation response directly by inhibiting mitochondrial metabolism with fewer side effects, which makes it a potential clinical radiosensitiser (53,54).

3.3 Oxygen mimetics as radiosensitisers

Oxygen mimetics, which are compounds developed with chemical properties of molecular oxygen with a better diffusion ability to low oxygen tissues, have also been explored for their radiosensitising proprieties (55). These include compounds such as misonidazole and nimorazole, which have been developed to mimic oxygen by promoting fixation of free radical damage during radiation (55). The use of misonidazole was halted at trial in combination with radiotherapy for treatment of inoperable squamous cell carcinoma of lung cancer due to its high toxicity, and a similar effect was observed in an investigation for treatment of advanced uterine carcinoma (56,57). Finally, the NIMRAD phase III trial explored the use of nimorazole in combination with Intensity-Modulated Radiotherapy (IMRT) in head and neck squamous cell carcinoma (HNSCC) (58) has been approved by the Centre for Clinical Practice (59).

4. Hypoxia-activated prodrugs as radiosensitisers

Hypoxia-activated prodrugs (HAPs) are compounds with high specificity for hypoxic tumours, as these are genotoxic compounds which are inactive in the presence of oxygen but are selectively activated under hypoxic conditions, and therefore can accurately target regions of tumour hypoxia (55). These HAPs have been identified and grouped into 5 main types: nitro

compounds, aromatic N-oxides, aliphatic N-oxides, quinones, and molecularly targeted HAPs (60). Nitro compounds-based HAPs include Metronidazole, PR-104A and TH-302, etc. The most representative N-oxide based HAPs are Tirapazamine, AQ4N and SN30000. Quinone-based HAPs, such as EO9 (Apaziquone), and Mitomycin C are the earliest developed hypoxia-activated prodrugs (61). Despite promising preclinical data of classical HAPs, limited clinical therapeutic efficacy have been shown in several HAPs, which led to the development of novel molecularly targeted HAPs in recent years, including CH-01 (hypoxia-activated Chk1/Aurora A inhibitor), TH-4000 (hypoxia-activated tyrosine kinase inhibitor), and CH-03 (hypoxia-activated KDAC inhibitor) (62-64). However, none of these have yet been evaluated in combination with radiotherapy. Details of HAPs being investigated in clinical trials as possible radiosensitisers of hypoxic cells as summarised in **Table 1**, with some examples detailed below.

4.1 Evofosfamide (TH-302)

TH-302 is an inactive compound of bromo-isophosphoramide which is released in hypoxic conditions and leads to alkylation of DNA (65). Interestingly, it has been shown that TH-302 in combination with radiotherapy enhances therapeutic outcomes (66). A further study also found that TH-302 has radiosensitising effects when administered in combination with a VEGF-A inhibitor in preclinical models of sarcoma, increasing DNA damage and apoptosis and decreasing HIF-1α activity (67). Further studies combining TH-302 and radiotherapy *in vivo and in vitro* reported a mild effect of treatment with TH-302 and a significant increase of apoptosis in hypoxic cells (68). However, a phase III clinical trial of TH-302 reported non-significant benefits and high toxicity, and therefore it has not been adopted clinically (69). There was a Phase I clinical trial using TH-302 with chemoradiotherapy in Oesophageal Cancer (NCT02598687) (70), however it was withdrawn as Phase II/III trials did not meet their primary endpoint, so further development and testing of TH-302 is uncertain.

4.2 Tirapazamine

Tirapazamine (TPZ) is an aromatic N-oxide which was first evaluated in 1986 and has been studied for its greater toxicity in anoxia when compared with aerobic conditions *in vitro (71).* TPZ specificity for hypoxic cells initially showed positive results in improving radiotherapy outcomes by using gene-directed enzyme prodrug therapy (GDEPT) in which hypoxia as a trigger for both enzyme expression and drug metabolism. (72). Preclinical *in vivo* studies in the early 90s had shown great promise. For example, a phase I clinical trial of TPZ in combination

with cisplatin and radiotherapy in SCLC (small cell lung cancer) leading to improved survival rate among patients, and a phase II clinical trial carried out on patients with locally advanced head and neck cancer reporting improved 3-year survival (73-75). Unfortunately, a later phase III clinical trial in locally advanced head and neck cancer showed no significant increase of patient survival (76).

4.3 AQ4N

Banoxantrone (AQ4N) is a bioreductive hypoxia-activated prodrug, which is bioreduced in hypoxic cells by cytochrome P450s (CYPs) to the cytotoxin AQ4 (77). Study found that AQ4N can selectively kill hypoxic cells via an inducible nitric oxide synthase (iNOS)-dependent mechanism when used in combination with radiation (78). Moreover, the use of AQ4N combined with radiotherapy and Temozolomide in glioblastoma entered a phase 2 clinical trial (NCT00394628), but no results have been published to date (79).

4.4 Mitomycin C (MMC)

Mitomycin C (MMC) is also a HAPs that generates DNA-damaging species via DNA cross-linking and has been shown to enhance toxicity against hypoxic compared to normoxic cells (60). Preclinical study revealed that MMC could enhance radio response and modulate hypoxic tumour microenvironment in combination with radiotherapy in rectal cancer (80). Clinical trials that used MMC combination with radiation are list in **Table 1**. Combined therapy includes 5-fluorouracil (5-FU), MMC and radiation has become current standard treatments of anal cancers and bladder cancers. RTOG-87-04 study Phase III randomized trial suggested that despite greater toxicity of MMC, the use of MMC can be beneficial, especially for those patients with large primary tumours (81). Long-term update of US GI intergroup RTOG 98-11 phase III trial compare chemoradiation therapy, replacing MMC with cisplatin due to the toxicity of MMC. However, cisplatin-based therapy failed to improve disease-free-survival compared with mitomycin-based therapy, therefore suggested RT + FU5/MMC remains the preferred standard of care of anal cancers (82,83).

5. Targeting of hypoxia-mediated signalling reprogramming as radiosensitising strategies

Targeting hypoxia-regulated signalling including and beyond direct HIF targeting in cancer has been explored as a therapeutic approach to reduce its tumour-promoting characteristics, and below we explore how targeting various hypoxia-regulated pathways can lead to improvement in radiotherapy responses (**Figure 2**).

5.1 HIF inhibition as a radiosensitiser strategy

As mentioned earlier, HIF is a critical factor in adaptation to the hypoxic microenvironment and is therefore an obvious molecular target to overcome radioresistance of hypoxic tumour cells (84). Several compounds have been studied as inhibitors of HIF-α transcription, translation, and protein stabilisation (85). Of these, some, such as SN-38 (the active metabolite of irinotecan), alongside its well established radiosensitiser effect as a topoisomerase I inhibitor, can also lead to increased radiosensitivity through inhibiting radiation-induced HIF-1α in colorectal cancer (86). T-type Ca^{2+} channel blockers, such as Mibefradil, which can block HIF-1 activation by reducing mitochondrial ROS production and increase HIF-1α protein hydroxylation and degradation (87), have also been studied in a clinical trial using Mibefradil with hypofractionated irradiation in recurrent GBM (88), with results suggesting that mibefradil can be safely co-administered with RT. STAT3 plays an important role in the response of tumour cells to radiotherapy, and STAT3 inhibitors NSC74859 and Stattic have been found to increase radiosensitivity by downregulating HIF-1α expression in oesophageal cancer (89-91). YC-1, a nitric oxide-independent activator of soluble guanylyl cyclase, was shown to enhance radiosensitivity across different types of cancer cells by inducing HIF-1α protein degradation and hence inhibition of HIF-1α function (92-94). More recently, other novel small-molecule inhibitors of HIF have been investigated. PX-478 decreases HIF-1α levels by inhibiting HIF-1α translation, as well as inhibiting de-ubiquitination leading to HIF-1α protein degradation (85). Palayoor and colleagues have shown a potential role for PX-478 as a clinical radiation enhancer in prostate carcinoma cells (95). HIF-2α inhibitors, including PT2399, PT2977, and PT2385, are also showing promise as single agents in ccRCC (clear cell renal cell carcinoma) in phase II clinical trials, but their combination with radiotherapy is not yet explored (96-98).

5.2 Targeting DNA Damage Response

Hypoxia can drive cancer progression and lead to radioresistance through its impact on genomic integrity by inhibiting DNA repair pathways (99). As outlined previously radiation

kills cancer cells by damaging their DNA. DNA repair dysregulation provide a promising opportunity to exploit this key vulnerability for overcoming radioresistance, specifically through targeting DSBs repair pathways (100). This is linked with the concept of 'synthetic lethality', which occurs when functional defects of complementary pathways can result in cell death, whereas the perturbation of either pathway does not impact cell survival Targeting one of the pathways using small molecule inhibitors in cells with a pre-existing defect in the complementary pathway (for example, use of PARP inhibitors in tumours defective for BRCA1/2) can be very effective, so other such pathway combinations have been explored (101-103). One of these is hypoxia-mediated repression of DNA repair in 'contextual synthetic lethality' approaches, for example through combination with PARP inhibitors (104). Finally, targeting of DNA Damage Response (DDR) key factors in combination with radiotherapy have shown a lot of potential for overcoming hypoxic radioresistance (105). Details of DDR inhibitors investigated in clinical trials as possible radiosensitisers is summarised in **Table 2**, and examples of these strategies are detailed below.

5.2.1 PARP1 inhibitors

PARP inhibitors (PARPi), which can effectively prevent the repair of damaged DNA by blocking PARP enzyme activity and PARylation reactions, are the first clinically approved drugs based on the principle of synthetic lethality (106). BRCA1/2 are major components of the HR (Homologous Recombination) pathway for DSB repair, and deficiency in BRCA1/2 genes leads to high susceptibility for breast and ovarian cancer (107). HR deficiency due to BRCA1/2 mutations leads to an exquisite sensitivity to PARPi through synthetic lethality between these two pathways, a phenomenon also described as BRCAness (108). Many clinical trials have been carried out in various BRCA-mutated tumours that have evaluated the benefits with the treatments of PARPi both as single agents and in combination with radiotherapy (109). Importantly, a study from 2010 reported that HR-defective hypoxic cells selectively died because of microenvironment-mediated "contextual synthetic lethality", where hypoxia-mediated repression of HR represented a BRCAness-like phenotype, and also enhanced sensitivity to ionising radiation (104). Other studies have also shown that the combination of PARP1 inhibitor Olaparib with radiotherapy led to radiosensitising effects in hypoxia in NSCLC through this contextual synthetic lethality effect (110). Moreover, PARPi also improves the radiotherapy responses, as well as the efficacy of some chemotherapeutic agents,

targeted therapy, and immunotherapy (111). This has led to a significant number of clinical trials focused on the combination with PARP1i and radiation to improve the response to radiotherapy (**Table 2**).

5.2.2 DNA-PK inhibitors

DNA DSBs generated by ionising radiation can also be repaired through NHEJ (Non-Homologous End Joining), a more error-prone repair pathway than HR (112). The KU heterodimers (KU70 and KU80) recognise the DNA DSBs, then activate and recruit DNA-PKcs to the DNA break sites. This complex formed at the DSBs consisting of DNA, Ku70/80, and DNA-PKcs is referred to as DNA-PK (113). The expression and activity of DNA-PK in cancers is correlated with the response to anticancer therapy, including radiotherapy (114). A study showed that DNA-PKcs inhibition led to increased sensitivity of gastric cancer cells to ionising radiation (115). Moreover, another study also found that DNA-PK inhibitor NU5455 may preferentially sensitise chronically hypoxic tumour cells to radiotherapy *in vivo* (116). Another study showed that DNA-PKcs inhibition potentially overcome hypoxia induced radioresistance in NSCLC by the combination of ionising radiation treatment with the DNA-PK inhibitor M3814 (117). To our knowledge, M3814 is the only DNA-PK inhibitor currently in clinical development (see Table 2).

5.2.3 ATM/ATR inhibitors

Ataxia-telangiectasia mutated (ATM) is one of the central kinases of the DDR and has a critical role in cancer suppression and DNA DSBs repair (118). Like ATM, Ataxia telangiectasia and Rad3 related (ATR) is also a central kinase involved in the DDR (119). Inhibition of ATM or ATR has been shown to sensitise the cancer cells to radiation treatments. Moreover, ATR and ATM have a role to play in hypoxia/re-oxygenation (120-122), which led to the exploration of ATM/ATR inhibitor treatment in overcoming hypoxia-mediated radioresistance in cancer. Inhibition of ATM or ATR has been shown to be potential radiosensitisers under hypoxic condition in several studies. One study found ATM inhibition can increase the radiosensitising effect under hypoxic conditions in non-small cells lung cancer (117). ATR inhibitor VE-821 has reported to increase sensitivity of pancreatic cancer cells to radiation and chemotherapy in pancreatic cancer under both normoxic and hypoxic conditions (120,123). Another ATR inhibitor from the same chemical series as VE-821, Berzosertib (formerly VE-822, M6620, and VX-970), has also been shown to sensitise response to chemo/radiotherapy, which could

improve the treatment efficacy in oesophageal cancer (124). Clinical trials regarding combination of ATM or ATR inhibitors with radiation are ongoing, such as ATM inhibitors AZD1390 and AZD6738, and ATR inhibitor VX-970 (**Table 2**). ATM and ATR target kinases CHK1 and CHK2 also represent attractive targets to be combined with established cancer therapies, including radiotherapy, but to date only CHK1 inhibitor Prexasertib/LY2606368 combined with radiation has entered clinical trial and suggest that this combination therapy may increase clinical benefit (125,126).

5.2.4 WEE1 Kinase Inhibitor

WEE1 kinase is a key regulator of the G2/M phase transition that allows DNA repair before mitotic entry (127). Amongst several WEE1 inhibitors evaluated in combination with radiotherapy (**Table 2**), combination of AZD1775 and ionising radiation has shown significantly increased apoptosis in cervical cancer cells (128). Another study also highlighted the radiosensitised effect of WEE1 Kinase inhibitor AZD1775 through inducing replication stress in hepatocellular carcinoma (129). Furthermore, another study investigated the impact of WEE1 inhibition using the MK-1775 on hypoxic cells in combination with radiation, showing MK-1775 sensitized radiation under normoxia, but not hypoxic conditions (130).

5.2 Targeting Cell metabolism

There are an increasing number of studies that conclude that metabolic alterations in cancer are one of the major reasons contributing to radioresistance (131). The PI3K/AKT/mTOR is a key signalling pathways that can stimulate glucose uptake, therefore controlling cell metabolism in cancer cells. The PI3K/AKT/mTOR pathway is involved in hypoxia-ischemia signalling, and HIF-1α is regulated by PI3K/Akt signalling pathway (132). PI3K inhibition by LY294002 radiosensitises human cervical cancer cell lines (133). Studies have also found that PI3K/Akt/mTOR pathway inhibitors (BEZ235 or PI103) enhance radiosensitivity in radioresistant tumour cells such as prostate cancer cells (134). A dual PI3K and mTOR inhibition NVP-BEZ235 have been shown to significantly reduce tumour hypoxia by normalizing tumour vasculature (135). PI3K/mTOR inhibitors BEZ235 and BKM120 were shown to significantly reduce oxygen consumption in cancer cell lines, with associated reduced mitochondrial respiration (136). Several clinical studies have now evaluated the efficacy of PI3K/Akt/mTOR inhibitors in combination with radiotherapy, and these are summarised in **Table 3**. Nelfinavir, which is AKT phosphorylation inhibitor, has entered

clinical trial phrase III in Cervical Cancer (137). Another study using Nelfinavir with concurrent CT-RT is associated with acceptable toxicity. Moreover, the results from metabolic response and tumour response suggested the benefit of nelfinavir is promising in stage IIIA/IIIB NSCLC (138).

Glucose transporter 1 (GLUT1) is an essential factor for glucose metabolism and is also a canonical HIF target gene (139). Studies found increased GLUT1 levels in radioresistant tumour cells, which indicates that GLUT1 expression may be used as an indicator of the sensitivity to radiation and prognosis of radiotherapy (140-142). Targeting GLUT1 and related signalling pathways may therefore represent an effective way to improve radiotherapy efficacy. A small molecule inhibitor of GLUT1, WZB117, can increase the sensitivity of radiation in breast cancer cells (143). Another study found that modulating the glucose metabolism sensitised Glioblastoma cells to ionising radiation (144). However, there are no GLUT1 inhibitors combined with radiation entered in clinic trails yet.

5.3 Combined Immunotherapy

During radiotherapy treatment, radiation not only damages cancer cells directly, but also activates an immune response (145). Meanwhile, hypoxia also plays a pivotal role in the regulation of immunosuppressive molecules and participates in the activation of immunosuppressive cells (146). For example, IL10 and TGFβ are increased under hypoxia, which induce the differentiation of tumour-associated macrophages (TAM) into M2 macrophages and therefore activates immune-suppressive activities (147). Hypoxia also regulates the differentiation and activation of dendritic cells (148). On the other hand, hypoxia activates immunosuppressive cells, such as myeloid-derived suppressor cells (MDSC), regulatory T cells and decreased infiltration and activation of cytotoxic T cells, which suggests that targeting HIF in the immune system could be beneficial for anti-tumour immune responses (149).

Radiotherapy has both pro-immunogenic and immunosuppressive effects on immune response in various levels. This includes the induction of immunogenic cell death, promoting the recruitment and function of T cells within the tumour microenvironment, and improving the recognition and killing of cancer cells by CD8+ cytotoxic T cells (CTLs) (150). This is key to the synergistic effect of radiation with immune checkpoint inhibitors, antibodies targeting inhibitory receptors on T cells, including cytotoxic T lymphocyte

antigen-4 (CTLA-4) and programmed death-1(PD-1), and has become an optimal partner for immune check point inhibitors (151). In fact, several completed clinical trials evaluated the efficacy of combining immunotherapy approaches using immune checkpoint inhibitors with radiotherapy, and the completed clinical trials are summarised in **Table 4**.

Furthermore, studies also found that immunosuppressive macrophages were recruited by radiation, which induced upregulation of CSF-1. Depletion of these macrophages by using anti-CSF antibody (aCSF) significantly delays tumour regrowth following radiation. Moreover, the addition of an anti-PD-L1 antibody (aPD-L1) to aCSF resulted in improved tumour suppression and even regression in a highly resistant murine pancreatic cancer model (152), therefore, macrophage depletion may play a role in immune checkpoint blockade-resistant tumours. Ultimately, as suggested by Franziska Eckert and colleagues, as hypoxia mediates radioresistance and immune escape, the combination of immune checkpoint inhibition and radiotherapy might be a promising strategy to improve outcome in tumours with high hypoxic content (153).

5.4 Intensity modulated radiation therapy (IMRT) combination with radiosensitiser approaches

IMRT is a radiotherapy modality that delivers highly conformal dose distributions (154). It is designed by inverse optimisation algorithms, with the following inputs: the dose required to the ‘tumour’ to gain control of the disease; and constraints or dose limitations for proximal tissues and ‘organs at risk’. The optimisation process is controlled by cost functions, these essentially compare dose distributions achieved by a set of x-ray beams, to the desired outcome; they then guide modulation of each beam in a systematic manner until a solution close to that originally specified is obtained. In simple terms the described process results in a set of beams, each consisting of a number of segments whose individual dose patterns superpose to create exquisite dose distributions that acknowledge the 3D nature of tumours and the discrete hypoxic and normoxic regions present in carcinogenic masses (155). Commonly, the degrees of freedom available to the optimiser is increased by using arc-based treatment beams rather than a discrete set of fixed directions. IMRT has had a clear impact on the success of modern radiotherapy strategies. However, given it typically is implemented with high energy x-rays which are low-LET (Linear Energy Transfer) radiation, further

developments considering strategy modification related to hypoxia management may be limited, see section 6.

Combining precise delivery via IMRT with radiosensitiser approaches such as DDR inhibitors (section 5.2) and immunotherapy (section 5.4) has the potential to improve patient outcomes. Furthermore, nanotechnology has potential to provide a new dimension to this strategy with metallic nanomaterials being developed as possible hypoxic radiosensitizers (156). Gold nanoparticles (GNP), for example, are gaining attention due to golds ability to readily donate electrons and thereby promote the production of reactive oxygen species, even in low oxygen environments. In a study of colon cancer, CT26 cells were incubated in hypoxia both with and without GNPs prior to radiotherapy application. Significantly improved responses were observed in the GNP group, suggesting dual IMRT-GNP therapeutics could improve the RBE and OER of low-LET modalities compared to x-ray application alone (157).

6 High-LET modalities as alternatives to oxygen-dependent low-LET ionising radiation

Linear energy transfer is the energy loss of a radioactive particle per unit of distance travelled and in radiotherapy, a measure of the amount of energy transferred from the radiation source to the patient. High-LET radiation sources include alpha particles, with high mass and positive charge, and low energy neutrons which have no charge and are approximately ¼ mass of an alpha particle (158). Low-LET radiation sources, most commonly x-rays or gamma-rays, are photons having no mass or charge and wavelengths below 10^{-8}m (159). High-LET particles deposit their energy within a short distance from the radiation source, following a discrete pathway and causing significant cellular disruption localised to a smaller area close to the target (160). Low-LET waves however penetrate tissues more readily and are widely scattered as they transverse through the patient, causing less intense damage to a larger area of tissue (160).

6.1 The radiobiology of high-LET RT modalities

Tumours with oxygen-deficient areas experience increased radioresistance termed the oxygen enhancement ratio (OER), a comparison of the dose of radiation needed to cause the same damage in normoxic verses hypoxic tissue environments. Experimentally, the OER is

inversely proportional to LET suggesting a potential clinical advantage of high-LET radiotherapy compared to low-LET irradiation (161). Relative biological effectiveness (RBE) is a comparison of biological efficacy of one type of ionising radiation compared to another (such as DNA damage and apoptosis levels), and indicates the dose of different ionisation sources that are needed to produce the same biological effect (159). High LET radiation has an increased biological effectiveness compared to photons of low LET, causing more extensive and clustered DNA damage (162). Specifically, application of high-LET radiotherapy causes closely interspaced DSBs leading to high local concentrations of repair proteins and perturbed DNA damage owed to its discrete pattern of energy deposition compared to low-LET X-ray irradiation (**Figure 3**) (163). Contemporary proton particle therapy utilises scanning beam technology which facilitate Intensity Modulated Proton Therapy, wherein the benefits afforded by Intensity Modulation and High-LET delivery are combined (164).

6.2 FLASH

FLASH radiotherapy is a treatment method that decreases the damage caused to the normal tissue (tissue sparing) whilst maintaining a tumour response compared with conventional low dose rate radiotherapy (165,166). The FLASH technique involves application of a single, ultra-high dose of radiation over a short time period. When compared to conventional radiotherapy *in vitro,* FLASH radiotherapy caused significantly less DNA damage to normal tissue than conventional radiation. The mechanisms underpinning the tissue sparing effect of FLASH is hypothesised to be diverse, including rapid radiochemical depletion of oxygen leading to transient hypoxia in normal tissue, radical too radical interaction, or inhibition of activation of genes that drive inflammation and proliferation of tumours (167). In the oxygen depletion/transient hypoxia hypothesis, normal tissue with physiological oxygen levels would experience rapid oxygen depletion after FLASH, leading to transient radioresistance which would in turn would lead to decreased damage and ultimately a tissue sparing effect. Further investigation on post irradiation effect showed that FLASH halted repopulation, whilst significantly reducing radio-induced senescence (168). Importantly, FLASH radiotherapy has increased RBE when delivered in high-LET modalities harnessing a proton beam radiation source compared to low-LET x-ray sources (169). Experiments to validate its efficacy in hypoxia however suggest FLASH radiotherapy has a high OER *in vitro,* with tissue oxygen concentrations above 4.4% needed for the technique to match the efficacy of conventional RT

as hypoxic regions lack the oxygen availability to support the rapid oxygen consumption occurring in local tissues during FLASH therapy (170). The mechanism and biological nature of the FLASH effect is complex, but it is expected this will be an area of increased interest in the radiobiology field.

6.3 Dose painting

Positron Emission Tomography (PET) and Magnetic Resonance Imaging (MRI) are functional, non-invasive imaging modalities utilised to identify hypoxic tissue regions in patient tumours (171). Such imaging allows clinicians to define areas likely to be resistant to radiotherapy, such as areas of tumour hypoxia. Therefore, strategic delivery of higher ionising doses to hypoxic areas while reducing the dose delivered to more oxygenated regions thereby limiting dose related side effects, a process also known as dose painting (172,173).

In a study of twelve patients with locally advanced HNSCC, hypoxia specific tracer ^{18}F-Fluoroazomycin arabinoside was harnessed alongside positron emission tomography technology (^{18}F-FAZA-PET) to assess the capabilities of hypoxia-guided dose painting. FAZA accumulation successfully identified hypoxic voxels in 80% of the cohort, while hypoxic volume made up to 54% of the patients' total tumour masses. Subsequently, 86 Gy doses were delivered to hypoxic voxels while a 70 Gy mean dose was administered across other regions and results revealed that dose escalation had no impact on adjoining healthy tissues [167]. Another dose painting study involving 10 HNSCC patients harnessed hypoxic tracer ^{18}F-Fluoromisonidazole in combination with PET (^{18}F-FMISO-PET) to identify and image chronic hypoxic voxels. Post imaging, one sub-group received 35 fraction schedules of 2Gy irradiation (70 Gy total) homogenously while a second sub-group received an escalated dose of 2.28 Gy to hypoxic regions (79.8 Gy total). Comparison of the two treatment plans demonstrated dose escalation to hypoxic regions can be delivered safely and efficaciously, without any increased delivery to at-risk organs [168]. Therefore, the literature suggests that combining dose-painting methodologies with high-LET radiation could therefore increase the benefit of hypoxia mapping as patients could benefit from the improved OER and RBE that high-LET therapies provide, accompanied by increased precision of application, allowing potent radiation doses to be delivered with minimal damage to healthy cells. However, a caveat of this approach is that it is based on a plan prior to treatment. A course of

radiotherapy is delivered over a period of one and seven weeks and oxygen level distribution can change in response to the treatment, thus impacting on the efficacy of this approach.

7 Concluding thoughts and future directions

Radiotherapy remains one of the most effective non-invasive treatments for solid tumours, but the impact of tumour biology on response of tumour cells to radiation remains a fundamental limitation to what radiotherapy can ultimately achieve. Challenges associated with radiotherapy response include inherent radioresistance of cancer cells, lack of discrimination between normal tissue and tumour cells, and, pertinent to this review, tumour hypoxia-mediated radioresistance. State-of-the-art dual treatment modalities for cancer patients have previously relied upon radiotherapy accompanied by surgery, chemotherapy and more recently, immunotherapy. However, these combinations have been unable to abolish treatment-resistant hypoxic regions often resulting in poor survival rates and disease recurrence. Furthermore, radiotherapy technology (instrumentation and software) and delivery has improved significantly over last 15 years, but has potentially encountered an era of diminishing returns, where increased accuracy in radiotherapy delivery may not substantially improve outcomes alone.

We suggest that hypoxia targeting in radiotherapy treatment strategies should encapsulate the mainstream treatment strategy for cancer, especially solid tumours, with experimental and clinical evidence suggesting some of these strategies even carry the benefit of reduced off-target effects. Of particular interest are treatment plans that strategically exploit the hypoxic tumour microenvironment by targeting hypoxia mediated radioresistance signalling, such as HIF inhibition and targeting DNA damage response, as well as employment of HAPs. However, further studies using accurate evaluation of hypoxic content of tumours is needed to validate their efficacy in combination with radiotherapy and advance such strategies towards the clinic.

Clinical validation of existing hypoxia targeted radiosensitisers should therefore continue to be a priority area in radiotherapy research, alongside prioritising treatment metrics that include hypoxic indices of tumours, capitalising on the disease-specific, druggable targets in the hypoxic microenvironment. This should include evaluating combination approaches of

radiotherapy with relevant hypoxia signalling targeting small molecule inhibitors (such as HIF and DDR inhibitors) as well as immunotherapy. These strategies should be also combined with current radiotherapy delivery modalities, including developing the use of hypoxia content scores in increasing the effectiveness of fractionated radiotherapy strategies using machine-learning in *in silico* modelling. It will also involve a shift towards high-LET radiotherapeutics over low-LET options to provide relatively immediate benefits to the cancer patient group.

Ultimately, the use of these various strategies targeting hypoxic radiobiology, combined with cutting-edge precise radiotherapy delivery and modelling, should lead to improvement in patient outcomes.

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Conflicts of Interest

None.

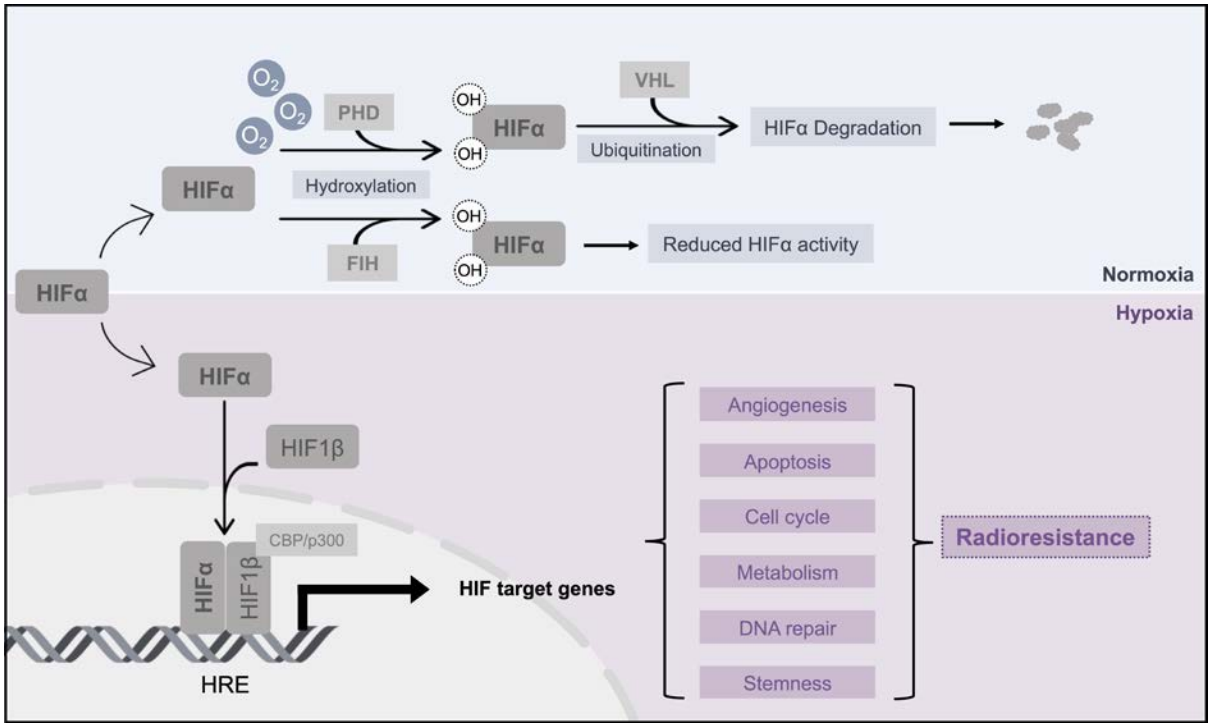

Figure 1. Mechanisms for HIF-α-mediated radiotherapy resistance

This schematic illustrates the key mechanisms for HIF stabilisation in hypoxic conditions, and highlights key pathways up-regulated by HIF that contribute to hypoxia-mediated radiotherapy resistance

HIF: Hypoxia-inducible factor; PHD: prolyl hydroxylases; FIH: Factor-inhibiting HIF; VHL: von Hippel-Lindau; OH: hydroxyl groups; CBP: CREB binding protein; HRE: hypoxia response elements.

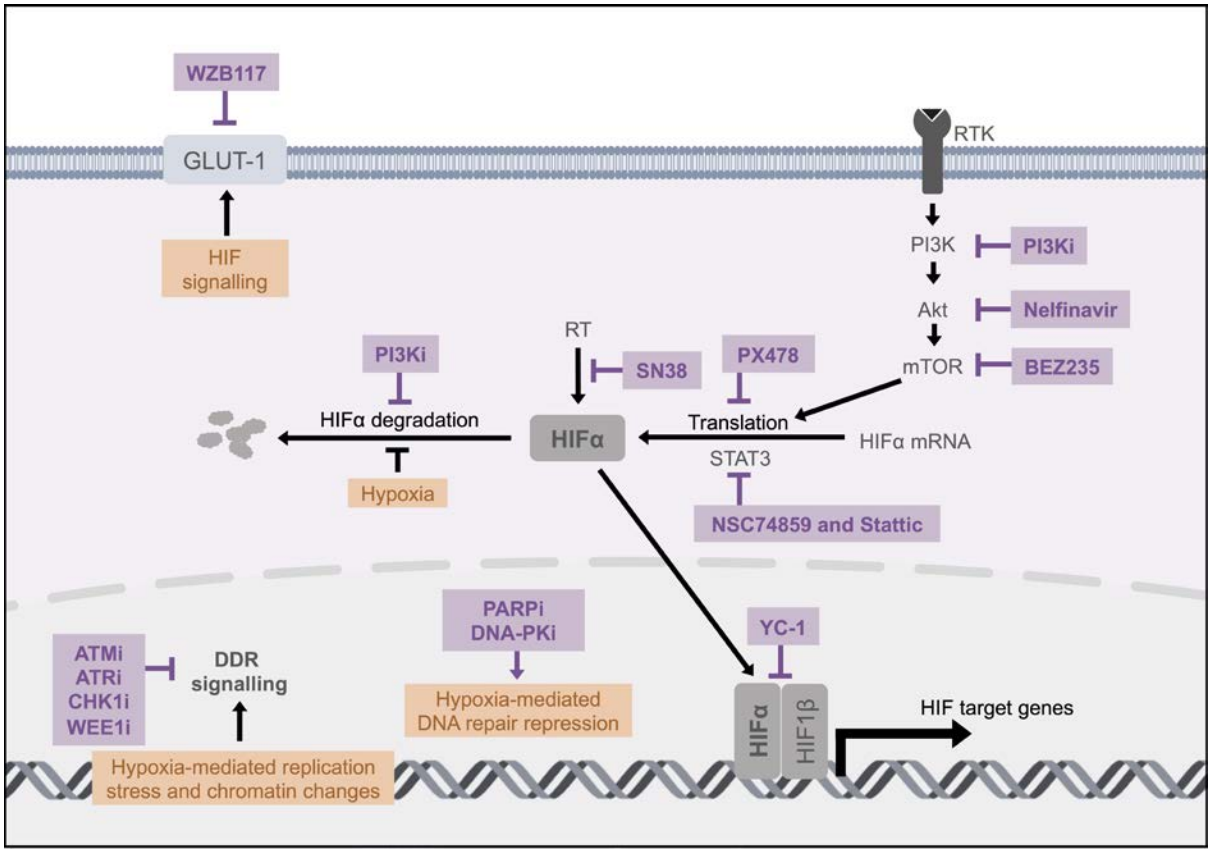

Figure 2. Targeting of hypoxia-mediated signalling reprogramming as radiosensitising strategies

This schematic indicates the key hypoxia-regulated or associated signalling pathways targeted in radiosensitising approaches, as detailed in section 5.

RT: Radiotherapy; HIF: Hypoxia-inducible factor; RTK: Receptor Tyrosine Kinases; DDR: DNA Damage Response; GLUT-1: Glucose Transporter 1; ATM: Ataxia Telangiectasia Mutated; ATR: Ataxia Telangiectasia and Rad3 related; CHK1: Checkpoint Kinase 1; PARP1: poly(ADP-ribose) polymerase.

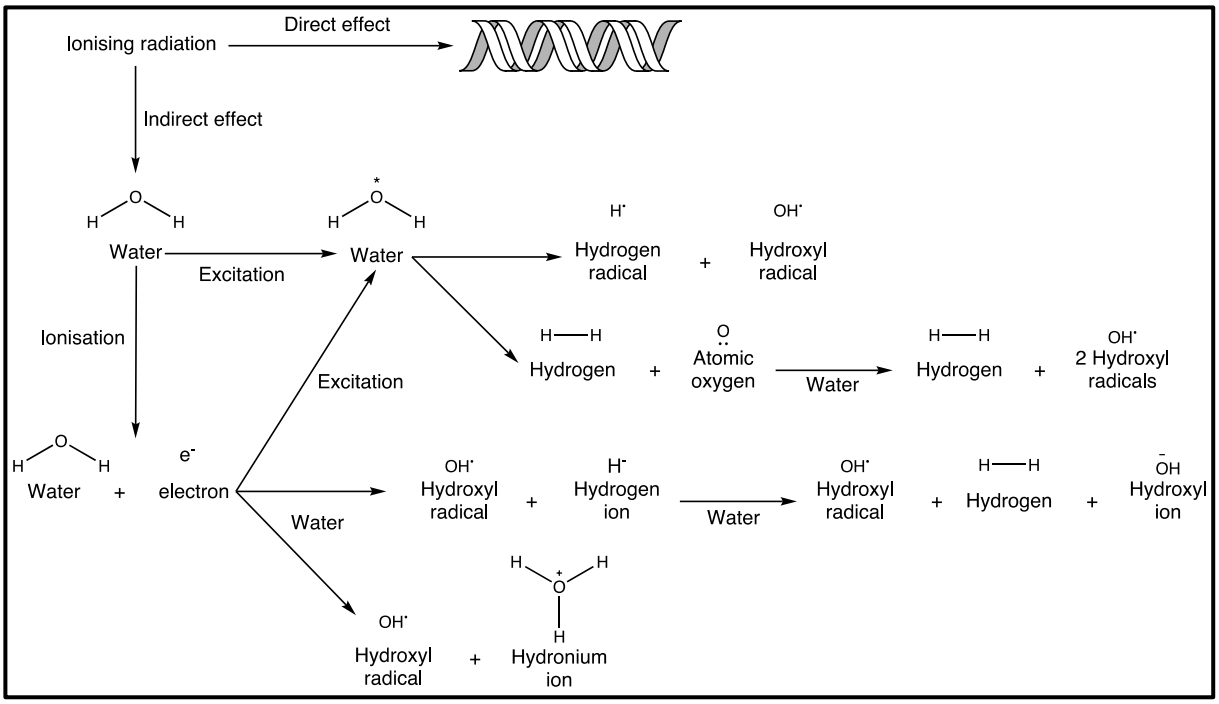

Figure 3. Water radiolysis in high vs low LET radiation

Water radiolysis, propagated by ionising radiation, can follow numerable reaction pathways resulting in a snowballing mechanism that produces numerable ROS (oxygen containing radicals) that damage DNA, known as the indirect effect. Additionally, radiation treatment can damage DNA through impact alone and subsequently damage molecular structure, known as the direct effect. Application of high-LET radiotherapy sources induce a larger degree of the direct effect compared to low-LET sources, owed to the particles high mass and charge, while low-LET modalities rely more on the presence of sufficient oxygen to be efficacious. Figure created in ChemDraw 20.1.1 and adapted from (174,175).

Table 1: Clinical trials evaluating combination of HAPs with radiotherapy

Drug Name	Cancer type	ClinicalTrials.gov Identifier	Clinical trial status (recruiting/active/completed)	References
Nitro Compounds				
Metronidazole	Cervical Cancer	NCT01937650	Phase II/III	(176)
Misonidazole	Head and Neck Cancer	NCT00606294	Not Applicable	(177)
Pimonidazole	Oral tongue cancer	NCT03181035	Phase I/II	(178)
Pimonidazole	Rectal Cancer	NCT02157246	Not Applicable	(179)
Etanidazole	Breast Cancer Brain Metastasis	NCT01985971	Not Applicable	(180)
Nimorazole	HNSCC	NCT01950689	Phase III	(181)
Nimorazole	HNSCC	NCT02661152	Phase III	(182)
Nimorazole	HNSCC	NCT01880359	Phase III	(183)
Nimorazole	HNSCC	NCT01733823	Phase I/II	(184)
Nimorazole	OSCC	NCT04124198	Not Applicable	(185)
N-Oxides				
Tirapazamine	SCCHN	NCT00002774	Phase II	(186)
Tirapazamine	Lung Cancer	NCT00066742	Phase II	(187)
Tirapazamine	Lung Cancer	NCT00033410	Phase I	(188)
Tirapazamine	Head and Neck cancer	NCT00094081	Phase III	(189)
Tirapazamine	Cervical Cancer	NCT00262821	Phase III	(190)
Tirapazamine	Cervical Cancer	NCT00098995	Phase I	(191)
Tirapazamine	HNSCC	NCT00174837	Phase III	(192)
Tirapazamine	Lung Cancer	NCT00006487	Phase I	(74)
AQ4N	Glioblastoma Multiforme	NCT00394628	Phase I/II	(193)
Quinones				
Mitomycin	Nasopharyngeal Carcinoma	NCT00201396	Phase III	(194)
Mitomycin	Pulmonary Neoplasm	NCT00128037	Phase II	(195)
Mitomycin	Bladder Cancer	NCT00002490	Phase III	(196)
Mitomycin	Bladder Cancer	NCT00024349	Phase III	(197,198)
Mitomycin	Bladder Cancer	NCT00981656	Phase II	(199)
Mitomycin	Head and Neck Cancer	NCT00002507	Phase III	(200)
Mitomycin	Anal Cancer	NCT00025090	Phase III	(201)
Mitomycin	Anal Cancer	NCT00003596	Phase III	(82,83)
Mitomycin	Anal Cancer	NCT01621217	Phase I	(202)
Mitomycin	Anal Cancer	NCT01941966	Phase II	(203)
Mitomycin	Anal Cancer	NCT02701088	Phase II	(204)
Mitomycin	Anal Cancer	NCT00423293	Phase II	(205)
Porfiromycin	Head and Neck Cancer	NCT00003328	Phase III	(206)
Porfiromycin	Head and Neck Cancer	NCT00002507	Phase III	(200)

HNSCC (Head and Neck Squamous Cell Carcinoma); OSCC (Oropharyngeal Squamous Cell Carcinoma); SCCHN (Squamous Neck Carcinoma of the Head and Neck Cancer)

Table 2. Clinical trials evaluating the combination of DDR inhibitors and radiotherapy

Drug Name	Cancer type	ClinicalTrials.gov Identifier	Clinical trial status (recruiting/active/ completed)	Strategies for combination with radiotherapy	References
PARP-1 inhibitors					
Olaparib	Inflammatory Breast Carcinoma	NCT03598257	Phase II	Radiation	(207)
Olaparib	TNBC	NCT03109080	Phase I	Radiation	(208)
Olaparib	GBM	NCT03212742	Phase I/IIa	IMRT, TMZ	(209)
Olaparib	NSCLC, breast cancer, HNSCC	NCT01562210 NCT02227082 NCT02229656	Phase I	Radiation, Cisplatin	(210)
Olaparib	NSCLC	NCT04380636	Phase III	Radiation, Etoposide, Carboplatin, Cisplatin, Paclitaxel, Pemetrexed, Durvalumab	(211)
Olaparib	Head and Neck Cancer	NCT02308072	Phase I	IMRT, Cisplatin	(212)
Olaparib	Prostate Cancer	NCT03317392	Phase I/II	Radium Ra 223 Dichloride	(213)
Olaparib	Extensive-Stage Small Cell Lung Cancer	NCT04728230	Phase I/II	Radiation, Carboplatin Durvalumab, Etoposide,	(214)
Veliparib	Peritoneal carcinomatosis	NCT01264432	Phase I	LDFWAR,	(215)
Veliparib	Brain metastases from NSCLC	NCT01657799	phase II	WBRT, Placebo	(216)
Veliparib	NSCLC	NCT02412371	Phase I	Radiation, Carboplatin, Paclitaxel	(217)
Veliparib	Rectal cancer	NCT01589419	phase I	Radiation, Capecitabine	(218)
Veliparib	Head and Neck Cancer	NCT01711541	Phase I/II	Radiation, Cisplatin, Carboplatin, Fluorouracil, Hydroxyurea	(219)
Veliparib	Cancer Patients with Brain Metastases	NCT00649207	Phase I	WBRT	(220)
Veliparib	Pancreatic Cancer	NCT01908478	Phase I	Radiation, Gemcitabine	(221)
Veliparib	Breast Cancer	NCT01477489	Phase I	Radiation	(222)
Veliparib	GBM	NCT01514201	Phase I/II	3D CRT, TMZ	(223)
Veliparib	GBM	NCT03581292	Phase II	Radiation, TMZ	
Veliparib	Lung Adenocarcinoma	NCT01386385	Phase I/II	3D CRT, Carboplatin, Paclitaxel	(224)
Niraparib	Prostate Cancer	NCT04194554	Phase I	SBRT, Leuprolide, Abiraterone Acetate	(225)
Niraparib	Metastatic Invasive Carcinoma of the Cervix	NCT03644342	Phase I/II	Radiation	(226)
Niraparib	TNBC	NCT03945721	Phase I	Radiation	(227)
Niraparib	Breast cancer	NCT04837209	Phase II	Radiation, Dostarlimab	
DNA PK inhibitors					
M3814	Advanced Solid Tumours	NCT02516813	Phase I	Radiation, Cisplatin	(228)
M3814	Rectal Cancer	NCT03770689	Phase I/II	Radiation, Capecitabine, Placebo	(229)
M3814	Solid Tumours	NCT03724890	Phase I	Radiation, Avelumab	(230)
M3814	GBM	NCT04555577	Phase I	Radiation, TMZ	(231)

M3814	HNSCC	NCT04533750	Phase I	Radiation	(232)
ATM/ATR inhibitors					
AZD1390	Brain cancer	NCT03423628	Phase I	Radiation	(233)
AZD6738	Solid tumours	NCT02223923	Phase I	Radiation	(234)
VX-970	HNSCC	NCT02567422	Phase I	Radiation, Cisplatin	(235)
VX-970	NSCLC brain metastases	NCT02589522	Phase I	WBRT	(236)
VX-970	Oesophageal Adenocarcinoma Squamous Cell Carcinoma Solid Tumor	NCT03641547	Phase I	Radiation, Cisplatin, Capecitabine	(237)
Elimusertib	Head and Neck Cancer	NCT04576091	Phase I	Radiation	(238)
WEE1 inhibitors					
AZD1775	Head and Neck Cancer	ISRCTN76291951 NCT03028766	Phase I	Radiation, Cisplatin	(239)
AZD1775	Adenocarcinoma of the Pancreas	NCT02037230	Phase I/II	Radiation, Gemcitabine	(240)
AZD1775	Cervical, Upper Vaginal and Uterine Cancers	NCT03345784	Phase I	Radiation, Cisplatin, Adavosertib	(241)
AZD1775	Cervical cancer	NCT01958658	Phase I	Radiation, Cisplatin	(242)
AZD1775	Head and Neck Cancer	NCT02585973	Phase I	Radiation, Cisplatin	(243)
AZD1775	GBM	NCT01849146 NCT01922076	Phase I	Radiation, TMZ	(244)

TNBC (Triple negative breast cancer); GBM (Glioblastoma); NSCLC (Non-small cell lung cancer); HNSCC (head and neck squamous cell carcinoma); IMRT(Intensity modulated radiotherapy), TMZ (Temozolomide); LDFWAR (low-dose fractionated whole abdominal radiation); WBRT (whole brain radiation therapy); 3D CRT (3-Dimensional Conformal Radiation Therapy); SBRT (Stereotactic body radiotherapy)

Table 3: Clinical trials evaluating the combination of PI3K/AKT/mTOR inhibitors and radiotherapy

Drug Name	Cancer types	ClinicalTrials.gov Identifier	Clinical trial status (recruiting/active/ completed)	Combination strategy	References
GDC-0084	Brain Metastases Leptomeningeal Metastasis	NCT04192981	Phase I	WBRT	(245)
GDC-0084	Brain and Central Nervous System Tumors	NCT03696355	Phase I	Radiation	(246)
GDC-0084	Glioma	NCT05009992	Phase II	Radiation, ONC201, Panobinostat	(247)
BKM120	NSCLC	NCT02128724	Phase I	Radiation	(135)
BKM120	HNSCC	NCT02113878	Phase I	IMRT, Cisplatin	(248)
Nelfinavir	Cervical Cancer	NCT03256916	Phase III	Radiation, Cisplatin	(137)
Nelfinavir	Locally Advanced Pancreatic Cancer	NCT03256916	Phase I	Radiation, Cisplatin, gemcitabine	(249)
Nelfinavir	NSCLC	NCT03256916	Phase I	Chemoradiotherapy	(138)
Nelfinavir	locally advanced rectal cancer	NCT03256916	Phase I	Chemoradiotherapy	(250)
Nelfinavir	Cervical Cancer	NCT01485731	Phase I	Radiation, Cisplatin	(251)
Nelfinavir	GMB	NCT00694837	Phase I	Radiation	(252)
Nelfinavir	Oligometastases	NCT01728779	Phase II	SBRT	(253)
Nelfinavir	Pancreatic Cancer	NCT01068327	Phase I	Radiation	(254)
BYL719	HNSCC	NCT02537223	Phase I	IMRT, Cisplatin	(255)
XL765	GMB	NCT00704080	Phase I	Radiation, TMZ	(256)
Alpelisib	Meningioma	NCT03631953	Phase I	MRI, Trametinib	(257)
Everolimus	Cervical Cancer	NCT01217177	Phase I	Radiation	(258)
Everolimus	Prostate Cancer	NCT01548807	Phase I	Radiation	(259)
Rapamycin	Rectum Cancer	NCT00409994	Phase I/II	Radiation	(260)
Temsirolimus	NSCLC	NCT00796796	Phase I	Radiation	(261,262)

GBM (Glioblastoma); NSCLC (Non-small cell lung cancer); HNSCC (head and neck squamous cell carcinoma); TMZ (Temozolomide); WBRT (whole brain radiation therapy); SBRT (Stereotactic body radiotherapy); IMRT (Intensity modulated radiotherapy)

Table 4: Clinical trials evaluating the combination of immunotherapy therapeutics and radiotherapy

Drug Name	Cancer types	ClinicalTrials.gov Identifier	Clinical trial (completed)	Combination with RT	References
Anti-PD-1/PD-L1					
SHR-1210	Oesophageal Cancer	NCT03187314	Phase I	Radiation	(263)
SHR-1210	Oesophageal Cancer	NCT03222440	Not Applicable	Radiation	(264)
Nivolumab	NSCLC	NCT02434081	Phase II	Radiation	(265)
Nivolumab	Small Cell Lung Cancer	NCT03325816	Phase I/II	Radiation	(266)
Nivolumab	Hepatocellular Carcinoma	NCT03380130	Phase II	SIRT	(267)
Nivolumab	Lung Cancer	NCT03044626	Phase II	Radiation	(268)
Pembrolizumab	Renal Cell Carcinoma	NCT02855203	Phase I/II	SABR	(269)
Pembrolizumab	Head and Neck Cancer	NCT02759575	Phase I/II	Radiation, Cisplatin	(270)
Pembrolizumab	Follicular Lymphoma	NCT02677155	Phase II	Radiation	(271)
Pembrolizumab	Metastatic Cancers	NCT02303990	Phase I	Radiation	(272)
Pembrolizumab	Oligometastatic Breast Neoplasia	NCT02303366	Phase I	SABR	(273)
Pembrolizumab	Esophageal Cancer	NCT02642809	Phase I	Radiation	(274)
Pembrolizumab	Renal Cell Cancer	NCT02599779	Phase II	SBRT	(275)
Nivolumab /Pembrolizumab	Lung cancer	NCT03224871	Phase I	Radiation, Intralesional IL-2	(276)
AMP-224	Colorectal Cancer	NCT02298946	Phase I	SBRT, Cyclophosphamide	(277)
Avelumab	NSCLC	NCT03158883	Phase I	SABR	(278)
Avelumab	GBM	NCT02968940	Phase II	HFRT	(279)
Cemiplimab	Advanced Malignancies	NCT02383212	Phase I	Radiation	(280,281)
anti-CTLA-4					
Ipilimumab	NSCLC	NCT02221739	Phase I/II	Radiation	(282)
Ipilimumab	Lymphoma	NCT02254772	Phase I/II	Radiation, SD-101	(283)
Ipilimumab	Melanoma	NCT01449279	Phase II	Radiation	(284)
Ipilimumab	Melanoma	NCT02406183	Phase I	SBRT	(285)
Ipilimumab	Melanoma, Brain Metastases	NCT02115139	Phase II	Radiation	(286)
Ipilimumab	Cervical Cancer	NCT01711515	Phase I	Radiation, Cisplatin	(287)
Tremelimumab	Pancreatic Cancer	NCT02311361	Phase I/II	SBRT, Durvalumab	(288)
Tremelimumab	Recurrent Small Cell Lung Carcinoma	NCT02701400	Phase II	SBRT, Durvalumab	(289)

GBM (Glioblastoma); NSCLC (Non-small cell lung cancer); IMRT(Intensity modulated radiotherapy), TMZ (Temozolomide); LDFWAR (low-dose fractionated whole abdominal radiation); WBRT (whole brain radiation therapy); 3D CRT (3-Dimensional Conformal Radiation Therapy); SBRT (Stereotactic body radiotherapy),SIRT (Selective internal radiation therapy); SABR (Stereotactic ablative radiotherapy); SBRT (Stereotactic Body Radiation Therapy); HFRT (Hypofractionated radiation therapy)

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