HHS Public Access

Author manuscript

Nat Rev Clin Oncol. Author manuscript; available in PMC 2023 February 01.

Published in final edited form as:

Nat Rev Clin Oncol. 2022 February ; 19(2): 114–131. doi:10.1038/s41571-021-00579-w.

Radiotherapy as a tool to elicit clinically actionable signalling pathways in cancer

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Abstract

A variety of targeted anticancer agents have been successfully introduced into clinical practice, largely reflecting their ability to inhibit specific molecular alterations that are required for disease progression. However, not all malignant cells rely on such alterations to survive, proliferate, disseminate and/or evade anticancer immunity, implying that many tumours are intrinsically resistant to targeted therapies. Radiotherapy is well known for its ability to activate cytotoxic signalling pathways that ultimately promote the death of cancer cells, as well as numerous cytoprotective mechanisms that are elicited by cellular damage. Importantly, many cytoprotective mechanisms elicited by radiotherapy can be abrogated by targeted anticancer agents, suggesting that radiotherapy could be harnessed to enhance the clinical efficacy of these drugs. In this Review, we discuss preclinical and clinical data that introduce radiotherapy as a tool to elicit or amplify clinically actionable signalling pathways in patients with cancer.

Over the past two decades, targeted anticancer agents have revolutionized the clinical management of a wide range of malignancies, largely reflecting the selective inhibition of aberrantly activated signalling pathways that are required for the survival, proliferation, dissemination and/or immunoevasion of cancer cells[1]. However, various tumours can be

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Author contributions

L.G. and S.C.F. conceived the article. G.P. and L.G. wrote the first version of the manuscript with critical input from L.C.C., L.S. and S.C.F. G.P. prepared display items under supervision from L.G. All authors approve the final version of the article.

Competing interests

L.S. has received research funding from Puretech. L.C.C. has acted as a consultant and/or advisor to Agios Pharmaceuticals, Faeth Therapeutics, Larkspur Therapeutics and Volastra Therapeutics, has received research funding from Petra Pharmaceuticals and is a co-founder of, and holds equity in, Agios Pharmaceuticals, Faeth Therapeutics, Larkspur Therapeutics and Volastra Therapeutics. S.C.F. has acted as a consultant and/or advisor to AstraZeneca, Bayer, Bristol Myers Squibb, Eisai, Elekta, EMD Serono, GlaxoSmithKline, Janssen, MedImmune, Merck US, Regeneron, Varian and ViewRay, and has received research funding from Bristol Myers Squibb, Eli-Lilly, Merck, Regeneron and Varian; and other support from Pfizer. L.G. has acted as a consultant and/or advisor to AstraZeneca, Boehringer Ingelheim, Inzen, The Longevity Labs, the Luke Heller TECPR2 Foundation, OmniSEQ and Onxeo, and has received research funding from Lytix, and Phosplatin. G.P. declares no competing interests.

Supplementary information

The online version contains supplementary material available at https://doi.org/10.1038/s41571-021-00579-w.

intrinsically resistant to targeted anticancer agents, because not all malignancies harbour genetic alterations that promote aberrant signal transduction (such as *KRAS* mutations), or because such signal transduction pathways emerge from epigenetic alterations or stress-responsive transcriptional programmes that are either not present or inactive at baseline[2], two situations that equally result in a lack of targetable alterations. In the former scenario, personalized, in-depth genetic characterization of the tumour might enable the identification of patients who are likely to benefit from targeted anticancer agents, such as those with non-small-cell lung carcinoma (NSCLC) driven by $KRAS^{G12C}$, who are now eligible to receive the $KRAS^{G12C}$-specific agent sotorasib (as second-line or later-line of therapy)[3]. A similar approach has also been successfully used in the latter scenario, for example by identifying patients with PD-L1^{+} tumours of various histologies, who are likely to benefit from immune-checkpoint inhibitors (ICIs)[4]. Moreover, at least in principle, cancer cells that lack a targetable alteration at baseline might become sensitized to certain targeted anticancer agents by harnessing the principle of ‘non-oncogene addition’, which involves rendering malignant cells dependent on otherwise non-oncogenic (and therapeutically actionable) signalling pathways[5].

More than 50% of patients with cancer receive radiotherapy as part of the clinical management of their disease either with curative intent (especially, but not exclusively in the context of early-stage disease[6,7]) or in palliative settings in order to contain the symptoms of metastatic disease, such as pain[8]. Radiotherapy is often used as a preoperative debulking intervention to facilitate surgical excision, as well as postoperatively (and less so intra-operatively) to control residual microscopic disease[9-11]. In all of these applications, radiotherapy has been demonstrated to reduce the incidence of local recurrence[9-11]. From a molecular standpoint, radiotherapy causes direct and reactive oxygen species (ROS)-dependent damage to DNA (and other molecules), potentially culminating in the permanent inactivation of cell division (cellular senescence) or the initiation of cell death programmes[12,13] (BOX 1). Intriguingly, cancer cells that succumb to radiotherapy also release abundant antigenic material as they emit immunostimulatory signals that support tumour-targeting immune responses[14]. Furthermore, accumulating preclinical and clinical findings suggest that the ultimate efficacy of radiotherapy might depend, at least in certain settings, on engagement of the patient’s immune system[15,16] (BOX 2).

Of note, the macromolecular damage imposed by radiotherapy affects both malignant and non-malignant cells, although the latter are relatively more radioresistant than the former as they can usually harness efficient repair mechanisms and are generally less proliferative[12]. Consistent with this notion, the acute adverse effects of radiotherapy tend to be more pronounced in non-malignant tissues with rapid cellular turnover, such as the gastrointestinal epithelium[17]. Thus, the terminal fate of irradiated cancer cells depends on their ability to successfully cope with the damage inflicted by radiotherapy by activating cytoprotective pathways that might enable avoidance of cellular senescence or regulated cell death, including (but not limited to): (1) proficient DNA damage resolution via the DNA damage response (DDR)[12]; (2) mitogenic signalling via surface-exposed receptors, such as HER2[18] and MET[19], or signal transducers thereof, such as PI3K[20] and MTOR[21]; (3) cellular stress management upon activation of macroautophagy (herein referred to as autophagy), which is an evolutionary conserved pathway for the preservation of cellular and organismal

homeostasis[22]; and (4) microenvironmental reconfiguration and immunoevasion, as driven by TGFβ[23] and PD-L1 signalling[24] (FIG. 1). Consistent with this notion, numerous targeted anticancer agents and ICIs have been tested for their ability to enhance the efficacy of radiotherapy[25,26]. Such a conceptual approach, however, has thus far achieved limited clinical success, potentially reflecting the notion that many tumours do not display non-oncogene addiction at baseline, but rather acquire such dependency during therapy through the emergence and/or expansion of treatment-resistant clones via positive selection[5,27]. This limitation suggests a crucial role for administration schedules in the efficacy of radiotherapy-containing therapeutic combinations[28].

Combining radiotherapy with targeted anticancer agents might be challenging in clinical settings, given that non-malignant tissues resist the detrimental effects of radiotherapy by harnessing the same cytoprotective mechanisms that support radioresistance (such as a proficient DDR)[12]. These overlapping mechanisms of resistance might explain the limited clinical success of such approaches when implemented according to standard-of-care (SOC) protocols that have been developed for each agent employed as standalone therapeutic interventions. This issue highlights the importance of a close collaboration between radiation oncologists and medical oncologists during trial design, with the aim of identifying a good compromise between efficacy and toxicity. For example, combining radiotherapy with an anti-PD-L1 antibody in patients with NSCLC creates a high risk of pneumonitis from each modality. Thus, investigators in a phase II trial with results published in June 2021 selected sub-ablative doses of preoperative stereotactic body radiotherapy (SBRT), which successfully avoided such pulmonary toxicities in patients with resectable NSCLC who were also receiving the anti-PD-L1 antibody durvalumab as a neoadjuvant therapy[29].

In this Review, we build on the pioneering work of Coleman and colleagues to further develop the innovative concept (originally introduced in 2013) of harnessing radiotherapy early in the course of treatment as a method for sensitizing malignant cells to targeted anticancer agents, thus expanding the range of oncological indications in which these drugs are effective[30]. As we summarize preclinical data supporting this novel use of radiotherapy, we discuss key aspects — including, but not limited to, administration schedules and potential for toxicities — for such a strategy to be successfully implemented in the clinic. We surmise that (at least for certain indications) this approach might improve the extent of local, and possibly systemic, disease control by enabling the early eradication of radiosensitive cancer cells, as well as the suppression of cancer cells that survive radiotherapy in the context of acquired radioresistance mediated by non-oncogene addiction.

DDR signalling

The best studied cellular effect of radiotherapy involves the direct and ROS-dependent formation of DNA single-strand and double-strand breaks (DSBs), which rapidly initiate the DDR as a cytoprotective response[31]. The primary goal of the DDR is to establish a reversible cell-cycle arrest that enables DNA repair and restoration of genomic integrity in cells harbouring damaged DNA[31]. In line with this notion, a proficient DDR is associated with reduced sensitivity to radiotherapy[12] and various DNA-damaging chemotherapeutic agents[32]. However, genomic instability generally supports malignant transformation and

tumour progression[33], and indeed tumours of various histologies are known to harbour deletions or loss-of-function mutations in various genes encoding components of the DDR, such as *BRCA1* and *ATM*[34]. These defects often elicit dependence on complementary non-oncogenic DDR pathways or accrued anti-apoptotic signalling, which has driven the development of various targeted therapies designed to harness the principle of synthetic lethality[31]. As a standalone example, poly(ADP-ribose) polymerase (PARP) inhibitors are now clinically approved drugs for use in patients with breast or ovarian cancers harbouring *BRCA1* or *BRCA2* mutations[35]. Along similar lines, acquired resistance to the DNA-damaging agent cisplatin has been shown to emerge alongside PARP1 hyperactivation and non-oncogene addiction to PARP1 (REF.[36]). Thus, DDR-targeting agents might also be effective in targeting malignant cells that acquire resistance to radiotherapy (Supplementary Table 1). Importantly, non-malignant cells are generally less sensitive to DNA-damaging agents than their malignant counterparts (reflecting, at least in part, their reduced proliferative rate)[12], which offers a therapeutic opportunity to combine radiotherapy with DDR-targeting agents in the context of acceptable toxicity (at least a priori).

ATM, ATR and DNA-PK inhibitors.

The serine/threonine kinase ATM has a major role in DSB repair upon irradiation, in part owing to the capacity to drive (at least initially) cytoprotective transcriptional programmes transduced by checkpoint kinase 2 (CHEK2) and orchestrated by p53 (REF.[37]). Consistent with this notion, germline *ATM* alterations, which lead to the human autosomal recessive disorder ataxia–telangiectasia, explain the extraordinary sensitivity of patients with ataxia–telangiectasia to ionizing radiation[38], as well as the inability of their lymphocytes to properly repair RT-induced DNA damage[39]. Moreover, basal ATM activation has been linked with radioresistance in stem-like cells isolated from patients with glioblastoma[40], and pharmacological ATM inhibition can boost the cytostatic and/or cytotoxic effects of radiotherapy on both human and mouse cancer cells in vitro[40-44] and in vivo[45-47], especially in the context of *TP53* mutations[45-47].

ATR is also involved in the repair of radiotherapy-induced DNA damage, although cytoprotective signals associated with ATR activation are mostly transduced via CHEK1, as opposed to CHEK2. ATR inhibition exacerbates the cytostatic and/or cytotoxic effects of radiotherapy in multiple preclinical tumour models[44,48-52], including patient-derived xenografts established from patients with triple-negative breast cancer (TNBC) recurring after chemotherapy[53]. Similar effects have also been documented with DNA-dependent protein kinase (DNA-PK) inhibitors, which target yet another component of the early DDR that can be activated by radiotherapy[54-56]. Of note, DNA-PK inhibitors appear to completely spare non-malignant tissues (at least in mouse models)[57] and therefore stand out as promising combinatorial partners for radiotherapy.

Importantly, radiotherapy can also synergize with ATM and ATR inhibitors via immunostimulatory mechanisms. In particular, both ATM inhibitors (such as KU60019) and ATR-targeting agents (such as ceralasertib) have been shown to: (1) stimulate type I interferon (IFN) release upon cyclic GMP–AMP synthase (CGAS) activation driven by the cytosolic accumulation of nuclear (as opposed to mostly mitochondrial, as in the case

of radiotherapy)[58] DNA fragments in preclinical models of breast, lung and pancreatic cancer[59-61]; and (2) abrogate the immunosuppressive effects of radiotherapy including the accumulation of $CD4^+CD25^+FOXP3^+$ regulatory T cells and PD-L1 expression by cancer cells in preclinical models of hepatocellular carcinoma (HCC) and colorectal cancer (CRC)[62,63]. These findings are in line with the notion that ATM drives immunosuppressive NF-κB signalling in radioresistant cancer cells[64], potentially linked to accrued genomic instability and indolent CGAS activation by micronuclei[65]. However, the observation that ATM silencing exacerbates PD-L1 overexpression driven by radiation in mouse models of pancreatic cancer, resulting in increased sensitivity to PD-L1 blockade, provides evidence to the contrary[61]. Whether this apparent discrepancy originates from an off-target effect of ceralasertib or the activation of compensatory pathways emerging from stable *ATM* knockdown remains to be elucidated.

Inhibitors of the MRN complex.

DSBs elicited by irradiation activate ATM via the so-called MRN complex, which encompasses meiotic recombination 11 (MRE11), DNA repair protein Rad50 (RAD50) and Nijmegen breakage syndrome 1 (NSB1)[66]. MRE11 dysfunction has been linked to enhanced tumorigenesis (independent of p53 and ATM) in a mouse model of oncogene-driven mammary carcinoma and to hypersensitivity to DNA-damaging agents as well as ATR, CHEK1 and PARP1 inhibitors in breast cancer cell lines and cancer stem cells derived from patients with CRC[67-69]. Consistent with this notion, women with TNBC expressing limited levels of MRN complex components (defined as <10% of nuclei staining for MRE11 or NBS1) have superior disease-specific survival than patients with abundant MRE11 or NBS1 expression[67], while overexpression of the MRN complex is correlated with a poor response to neoadjuvant radiotherapy in patients with rectal cancer[70]. Moreover, the transgene-driven expression of a RAD50 variant that weakens the interactions between MRN components has been shown to enhance the sensitivity of nasopharyngeal cancer cells to radiation, both in vitro and in vivo[71]. However, high MRE11 levels (defined as >25th percentile) are also predictive of improved overall survival (OS) in patients with muscle-invasive bladder cancer receiving radiotherapy[72]. Whether this latter observation reflects the ability of MRE11 to drive type I IFN signalling in response to cytosolic DNA[73] remains to be clarified. Irrespective, MRE11 can be cleaved into an inactive variant that lacks nuclease and DNA-binding activities but still assembles with the MRN complex in response to histone deacetylase (HDAC) inhibitors[74]. Consistent with a key role for MRE11 in DNA repair following radiotherapy, various HDAC inhibitors, including the clinically approved agents panobinostat and romidepsin, have been shown to disrupt the DDR and synergize with radiotherapy without notable increases in systemic toxicity in various xenograft models of urothelial carcinoma[75,76].

PARP inhibitors.

PARPs are a superfamily of DNA repair enzymes with functions that are essential for the survival of cancer cells bearing homologous recombination defects, such as those imposed by loss-of-function *BRCA1* and *BRCA2* mutations[77]. Various PARPs, including PARP1, are recruited to radiotherapy-induced DSBs and activate the DDR by promoting DNA end resection via the MRN complex[78], and rapidly decondensing chromatin at sites of DNA

damage[79], pointing to a potential synergy between PARP inhibitors and radiotherapy in the control of malignant cells. Consistent with this notion, various FDA-approved and experimental PARP inhibitors, including olaparib[80-83], talazoparib[84], veliparib[82,85] and fluzoparib[86], are able to interfere with the cytoprotective DDR elicited by radiation in cancer cells, resulting in increased DSB accumulation and apoptotic cell death. Similarly, combined PARP and ATR inhibition has profound radiosensitizing effects in patient-derived glioblastoma stem-like cells (which have highly proficient DDR) by abrogating the G2–M arrest promoted by radiotherapy, ultimately driving malignant cells towards mitotic catastrophe[87]. While PARP inhibitors are currently approved as monotherapies for patients with *BRCA1*-mutant and *BRCA2*-mutant cancers, preclinical evidence supports the possibility that radiotherapy can be harnessed to either elicit or aggravate non-oncogene addiction to PARP in both the presence and absence of functional BRCA1/2 proteins[81-83,86], as well as in malignant cells lacking polybromo 1 (*PBRM1*), which encodes a chromatin remodelling enzyme affected by loss-of-function mutations in 40% of clear cell renal cell carcinomas[88]. Of note, while combining radiotherapy with PARP inhibitors is expected to be effective in homologous-recombination-incompetent tumours, reliable tools to detect such a ‘BRCAness’ phenotype in malignancies bearing functional BRCA1 and BRCA2 are missing[89]. Conversely, identifying tumours that utilize alternative non-homologous end-joining for DSB repair in the absence of homologous recombination stands out as a promising tool to predict sensitivity to radiotherapy plus PARP inhibitors[90].

Intriguingly, multiple FDA-approved PARP inhibitors (such as olaparib, rucaparib and niraparib) have been shown to promote robust CGAS-dependent type I IFN secretion by malignant cells following the cytosolic accumulation of nuclear DNA or micronuclei, thus promoting anticancer immunity[91]. Radiotherapy largely drives type I IFN secretion via mitochondrial (rather than nuclear) DNA[58], suggesting that PARP inhibitors might robustly boost the tumour-targeting immune response driven by radiotherapy (BOX 2). Accordingly, niraparib has been shown to improve radiation-induced $CD8^+$ T cell activation in a mouse model of *EGFR*-mutant NSCLC, correlating with superior CGAS signalling in malignant cells[92]. Both PARP inhibitors and radiotherapy have been linked with PD-L1 upregulation in certain settings[16,93], implying that the addition of ICIs stands out as a potential strategy to further improve the therapeutic efficacy of this combination.

Inhibitors of cell-cycle checkpoint kinases.

CHEK1, CHEK2 and WEE1 G2 checkpoint kinase (WEE1) are also involved in the DDR. However, while WEE1 phosphorylates cyclin-dependent kinase 1 (CDK1) to prevent mitotic entry, CHEK1 and CHEK2 mainly act as downstream effectors of ATR and ATM, respectively, to block the transition from the G1 to the S phase of the cell cycle[12]. WEE1 and CHEK1 are upregulated by radiation, not only in vitro[94-96], but also in patients with disease recurrence following radiotherapy, as demonstrated in patients with radioresistant human papillomavirus-positive (HPV^+) head and neck squamous cell carcinoma (HNSCC)[97]. Consistent with this notion, experimental inhibitors of WEE1 (REFS[98-100]) and CHEK1/2 (REFS[101-104]) have been shown to compromise cellular adaptation to radiotherapy by abrogating activation of the irradiation-induced G2 checkpoint, thus promoting mitotic catastrophe, an effect that is further aggravated by PARP inhibition[105-107]. Of note, CHEK1

inhibitors might also synergize with radiotherapy by compromising the formation of RAD51 nuclear foci, which is critical for homologous recombination[108,109].

The orally available pan-CDK inhibitor AZD5438 has been shown to mediate considerable radiosensitizing effects on radioresistant NSCLC cell lines, both in vitro and in vivo[110]. Nonetheless, more attention has been focused on specific CDK4/6 inhibitors (that inhibit cell-cycle progression at the G1–S transition), especially following the approval of palbociclib, ribociclib and later abemaciclib (all in combination with an aromatase inhibitor) as first-line therapies for patients with advanced-stage and/or metastatic oestrogen-receptor-positive (ER^+), $HER2^-$ breast cancer[111,112]. A growing body of preclinical literature demonstrates that these agents can be successfully combined with radiotherapy, resulting in delayed repair of radiation-induced DNA damage[113-115], prolonged cell-cycle blockade[116], and enhanced apoptosis[117], especially (but not exclusively) in models that retain p53 function[114,116,118]. Importantly, research involving xenograft models of glioblastoma and atypical teratoid rhabdoid tumour[119] as well as immunocompetent models of ER^+ breast cancer and TNBC[116], has demonstrated improved tumour control when palbociclib is administered after completion of (rather than before or concomitant with) hypofractionated radiation. Similar findings have been obtained with abemaciclib in a study involving several human NSCLC cell lines that were either maintained in vitro or xenografted into immunodeficient mice[114]. These data support the notion that cancer cells that can escape the G2–M arrest are selected for by radiotherapy and that further disease progression in this malignant cell population can then be inhibited using CDK4/6 inhibitors, thus highlighting the critical importance of treatment schedule for optimal therapeutic effects.

Intriguingly, multiple agents that interfere with cell-cycle progression can also mediate immunomodulatory effects that can be maximized by radiotherapy. For example, adavosertib (a first-in-class, orally available WEE1 kinase inhibitor) reportedly enhances the $CD8^+$ T cell responses driven by single-fraction radiation against a variety of tumours in immunocompetent mouse models[120,121]. However, although this combination seems to promote PD-L1 expression (suggesting benefit from the addition of ICIs)[120], the extent of tumour shrinkage was found to correlate with PD-L1 downregulation in immunocompetent models of breast cancer[121]. Whether this apparent discrepancy reflects variations in radiation dose (8 Gy versus 12 Gy) or intrinsic tumour features currently remains unclear. A similar radiation-enhanced antitumour immune response has been obtained with the experimental CHEK1/2 inhibitor AZD7762 followed by single-dose (17 Gy) irradiation in an immunocompetent mouse model of melanoma[122]. In this setting, AZD7762 followed by irradiation (but not either intervention alone) resulted in micronucleation and abundant secretion of type I IFN by cultured malignant cells, eliciting robust systemic anticancer immune responses when tested in vivo[122]. Whether delivering AZD7762 after radiotherapy would further enhance the immunotherapeutic effects of the combination therapy remains to be investigated. Along similar lines, despite an abundant preclinical literature suggesting that radiotherapy and CDK4/6 inhibitors stand out as promising combination partners owing to their ability to activate poorly overlapping immunostimulatory pathways[123], mechanistic evidence supporting this possibility is currently lacking.

Clinical considerations.

PARP inhibitors provide one of the first validations of the clinical utility of synthetic lethality[124]. Specifically, PARP inhibitors are not only approved as monotherapies for use in patients with breast, ovarian or prostate cancer harbouring loss-of-function *BRCA1, BRCA2* or *ATM* mutations[125,126], but have been and are being extensively tested in combination with various treatment strategies including chemotherapy, radiotherapy and immunotherapy. Monotherapy with PARP inhibitors is generally well tolerated, with common toxicities including myelosuppression, gastrointestinal symptoms and fatigue, albeit with a low risk (<1%) of secondary malignancies owing to DNA[12,127]. Similarly, standard-dose radiotherapy administered in combination with olaparib has demonstrated an acceptable safety profile in patients with locally advanced or metastatic HNSCC or TNBC[128,129]. Conversely, olaparib combined with higher-dose radiotherapy and concurrent cisplatin was poorly tolerated by patients with locally advanced NSCLC, resulting in severe oesophageal and haematological toxicities, as well as pulmonary adverse events[130]. While conformal radiotherapy schedules and techniques enabling improved pulmonary and oesophageal sparing should be implemented in order to further explore therapeutic combinations involving PARP inhibitors, other strategies can also enhance efficacy. For example, preliminary clinical evidence demonstrates that olaparib combined with alpelisib (an FDA-approved α-specific PI3K inhibitor) or buparlisib (an orally available experimental broad-spectrum PI3K inhibitor) is well tolerated and has synergistic effects in patients with advanced-stage and/or recurrent ovarian or breast tumours[131,132]. The safety and efficacy of adding radiotherapy to this combination, however, remains to be clinically investigated.

Unlike PARP inhibitors, ATM, ATR, DNA-PK, WEE1 and CHEK1 inhibitors are still in early-phase clinical development and have largely not been investigated in combination with radiotherapy. Berzosertib, a first-in-class ATR inhibitor previously assessed for safety and preliminary efficacy as monotherapy or combined with various chemotherapies in patients with advanced-stage solid tumours[133,134], is currently being investigated in combination with radiotherapy in patients with brain metastases from NSCLC (NCT02589522), in those with chemotherapy-resistant breast cancer (NCT04052555), and (combined with cisplatin) in those with locally advanced HNSCC (NCT02567422). Similarly, the ATR inhibitor ceralasertib[135–136] (NCT02223923) is currently being tested in combination with radiotherapy in patients with advanced-stage solid tumours, while the DNA-PK inhibitor peposertib (formerly known as nedisertib)[137] is currently being investigated in combination with radiotherapy and SOC chemotherapy in at least nine phase I/II basket trials (NCT02516813, NCT03724890, NCT03770689, NCT04068194, NCT04071236, NCT04172532, NCT04533750, NCT04555577 and NCT04750954). Preliminary evidence suggests that both the WEE1 inhibitor adavosertib and the CHEK1 inhibitor prexasertib are well tolerated when combined with radiotherapy or chemoradiotherapy in patients with pancreatic cancer[138] and in those with advanced-stage HNSCC[139], resulting in several phase I/II clinical trials testing similar regimens (NCT02585973, NCT02555644, NCT03028766 and NCT04460937). The final results of these studies, however, have thus far not been reported.

The clinical development of AZD5438 and AZD7762 has been discontinued owing to exposure and/or tolerability issues[140,141]. Conversely, data from several cohorts of patients with metastatic ER^+ breast cancer who received CDK4/6 inhibitors suggest that concomitant administration of CDK4/6 inhibitors with radiotherapy does not significantly increase the incidence of neutropenia relative to that observed with CDK4/6 inhibitors alone[142-146]. Other notable toxicities include sporadic episodes of high-grade but reversible intestinal toxicities when the bowel is located within the radiation field[147]. Similarly, radiotherapy combined with ribociclib is well tolerated with preliminary signs of clinical activity in children with newly diagnosed diffuse intrinsic pontine glioma[148]. Importantly, preclinical findings from our research group and others demonstrate that treatment schedule is a major determinant of efficacy when radiation is combined with CDK4/6 inhibitors[116,119]. These findings inspired the design of a randomized phase II trial comparing the efficacy of palbociclib plus letrozole versus the same regimen preceded by SBRT in patients with oligometastatic (five or fewer metastases) ER^+ $HER2^-$ breast cancer (NCT04563507). Various other phase I/II trials assessing the role of radiotherapy in combination with palbociclib, ribociclib or abemaciclib in patients with breast cancer (NCT03691493, NCT04334330, NCT04605562 and NCT04923542) or in those with other solid tumours, including HNSCC (NCT03024489), glioma (NCT03355794) and prostate cancer (NCT04298983), are ongoing.

PI3K signalling

The PI3K signal transduction cascade is the most frequently dysregulated pathway in human cancer[149]. PI3K, which exists in several isoforms, is generally activated by receptor tyrosine kinases (RTKs) or G-protein-coupled receptors and promotes the phosphorylation-dependent activation of AKT serine/threonine kinase 1 (AKT1). In turn, active AKT1 can phosphorylate a variety of substrates that promote cellular survival and anabolism including MTOR, thus supporting cellular proliferation[20]. Multiple mechanisms can lead to aberrant PI3K signalling in malignant cells: (1) genetic alterations affecting specific PI3K-coding genes such as phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit-α (*PIK3CA*) as well as *AKT1, MTOR,* and phosphatase and tensin homologue (*PTEN*), which encodes a prominent PI3K antagonist[149-151]; (2) genetic or epigenetic defects that culminate in the overexpression or hyperactivation of oncogenic RTKs, including EGFR, HER2, MET and KIT[149,152]; and (3) additional features such as the composition of the local microbiome, which has been linked with PI3K activation in patients with lung cancer[153] and dysregulated insulin signalling, pointing to a role for insulinaemia-controlling strategies including metformin (an FDA-approved drug for type 2 diabetes mellitus) and ketogenic diets as promising partners for combination with PI3K inhibitors[154,155]. Considerable efforts have been dedicated to the development of PI3K inhibitors for clinical use, culminating in the approval of alpelisib in combination with fulvestrant for use in patients with advanced-stage and/or metastatic ER^+ $HER2^-$ breast cancer harbouring *PIK3CA* mutations[156].

Extensive evidence links PI3K hyperactivation to the emergence of radioresistance. For example, activating *PIK3CA* mutations have been associated with an increased risk of local treatment failure in patients undergoing SBRT for primary or metastatic lung lesions[157] and in those receiving whole-brain radiotherapy for brain metastases[158]. Similarly,

hyperactivation of AKT1 downstream of *PTEN* loss has been associated with an elevated risk of relapse after radiotherapy in patients with prostate cancer harbouring copy number increases in *MYC*[159]. Moreover, patients with HNSCC or nasopharyngeal carcinoma with high levels of EGFR expression[160,161], women with HER2$^+$ breast cancer[162], those with locally invasive prostate cancer staining positively for vascular endothelial growth factor A (VEGFA)[163], and patients with uterine cervical cancer harbouring alterations in *FGFR1, FGFR2, FGFR3* and *FGFR4* (REFS[164,165]) have high rates of disease recurrence following radiotherapy. Finally, radiotherapy can drive cytoprotective PI3K signalling by promoting the upregulation or activation of various upstream activators of PI3K, including (but not limited to) EGFR[166-169], HER2 (REF.[170]), MET[171-174], VEGFR2 (REFS[175-178]) and FGFR2 (REF.[179]), which has been consistently associated with radioresistance coupled with the acquisition of mesenchymal and stem-like features by malignant cells[180-183]. Thus, multiple nodes of the PI3K signal transduction cascade stand out as promising targets for interventions designed to overcome acquired resistance to radiotherapy[184,185] (Supplementary Table 2).

PI3K, AKT and MTOR inhibitors.

An abundant body of preclinical literature demonstrates that pharmacological inhibition of PI3K synergizes with radiation in a variety of tumour models. For example, the orally available PI3K/MTOR inhibitor dactolisib has been shown to substantially increase the radiosensitivity of human prostate cancer cells in vitro, correlating with increased biomarkers of an epithelial (over mesenchymal) phenotype[182]. Similar findings have been obtained with the poorly selective PI3K inhibitor LY294002 (REFS[167,186,187]) and the orally available PI3Kα/δ inhibitor pictilisib[188] in models of human high-grade glioma, especially when combined with radiation and temozolomide (which mimics the SOC for patients with glioblastoma)[189]. Moreover, irradiation has been shown to confer improved tumour control in immunocompromised mice bearing human pancreatic tumours[190] or HNSCCs harbouring *PIK3CA* mutations[191] when combined with the PI3K inhibitors HS-173 or taselisib, respectively. Conversely, neither the pan-PI3K inhibitor buparlisib nor the dual PI3Kα/β/δ/γ and MTOR inhibitor apitolisib mediates radiosensitizing effects in cultured HNSCC cells, despite effective PI3K inhibition[192]. These findings suggest that, at least in certain cell types, compensatory mechanisms might preserve the radioresistant phenotype despite adequate PI3K inhibition. Supporting this possibility, the experimental AKT1 inhibitor MK-2206 has been shown to block AKT1 signalling in *PTEN*$^{-/-}$ glioblastoma cells but failed to improve the therapeutic activity of radiotherapy owing to aberrant downstream activation of MTOR[193]. Consistent with this notion, dactolisib demonstrated excellent synergy with radiation in preclinical models of endometrial cancer[194], an effect that was linked with reduced PI3K, MTOR and VEGFA activity[194,195]. Moreover, pharmacological inhibition of AKT1 or MTOR after (but not before) fractionated irradiation has been shown to enhance the loss of clonogenicity of cultured radioresistant human prostate cancer cells[196]. Finally, co-administration of the MTOR inhibitor vistusertib with buparlisib or alpelisib appears to sensitize radioresistant human oral squamous cell carcinoma cells to radiation[197]. Whether similar effects can be achieved in immunocompetent preclinical models remains largely uninvestigated. Irrespective of these and other unknown aspects, PI3K inhibition synergizes with PARP inhibitors in preclinical models of BRCA1-defective

and p53-defective breast cancer[198], PTEN-deficient and p53-deficient prostate cancer[199], and PTEN-deficient endometrial cancer[200,201], suggesting that concurrent inhibition of PARP and PI3K in combination with radiotherapy is a promising therapeutic approach.

RTK inhibitors.

Pharmacological agents targeting EGFR (such as AG1478)[167] as well as expression of a dominant-negative form of the type III EGFR variant (EGFRvIII)[202] reportedly increase the radiosensitivity of glioblastoma cells, correlating with abrogated AKT1 phosphorylation. Similar data have been obtained with clinically available anti-EGFR monoclonal antibodies (nimotuzumab and cetuximab) in human NSCLC cells growing in vitro or xenografted into immunodeficient mice[203,204]. Moreover, treatment with the dual EGFR/HER2 tyrosine-kinase inhibitors lapatinib or pyrotinib reduces HER2 phosphorylation and downstream AKT1 activation, ultimately sensitizing $HER2^+$ breast and gastric carcinoma cells to radiation[205,206]. HER2 has also been shown to mediate AKT1-dependent immunosuppressive effects including the suppression of cytosolic DNA sensing and consequent abrogation of CGAS signal transducer stimulator of IFN response cGAMP interactor 1 (STING1) signalling[207], as well as upregulation of the phagocytosis inhibitor CD47 (REF.[208]). Accordingly, dual blockade of CD47 and HER2 has been demonstrated to maximize macrophage-dependent phagocytosis and promote the eradication of radioresistant breast cancer cells[208].

Cediranib, an orally available VEGFR inhibitor, limits radiation-induced VEGFR phosphorylation in endothelial cells, thus sensitizing lung cancer and CRC xenografts to fractionated radiation, at least partly owing to aggravated vascular disruption[209,210]. Similarly, apatinib (a selective VEGFR2 inhibitor also known as rivoceranib) promotes radiation-induced cell death in HCC xenografts, largely via suppression of radiation-induced PI3K signalling[211]. Moreover, genetic inhibition of VEGFA exacerbates the extent of DNA damage in irradiated nasopharyngeal carcinoma cells via a mechanism that involves compensatory activation of MTOR signalling and inhibition of autophagy, a cytoprotective pathway that (among other functions) supports DNA repair[212]. The pan-FGFR inhibitors LY2874455 and AZD4547 have been shown to sensitize multiple cancer cell lines and two human NSCLC xenografts to carbon ion and conventional radiation, respectively[165,213], correlating with decreased AKT1 phosphorylation in vitro[213]. Finally, inhibition of MET with the FDA-approved agents crizotinib[173] and tepotinib[174], as well as with the experimental ATP-competitive inhibitor JNJ38877605 (REF.[214]), has been shown to improve the efficacy of radiation in mouse models of HNSCC and glioblastoma, an effect that was linked to the ability of MET inhibitors to overcome the enrichment of radioresistant stem-like cells that tend to occur in the context of sub-ablative doses of radiation.

In vivo data obtained from mouse fibrosarcoma cells growing in immunocompetent hosts demonstrate that optimal tumour control is achieved when anti-VEGFR2 antibodies are delivered shortly before (but not shortly after) irradiation, owing to interception of very rapid-onset VEGFA signalling driven by irradiation[215]. Along similar lines, AZD4547 ameliorates the radiosensitivity of glioma neurospheres orthotopically implanted into immunodeficient mice, albeit only when FGFR2-driven DNA repair, involving nuclear

PTEN phosphorylation, is successfully inhibited[179]. These data lend further support to the critical importance of treatment schedule for the therapeutic efficacy of combination regimens involving radiotherapy.

Clinical considerations.

Over the past three decades, a large number of drugs targeting PI3K, its activators or its effectors have been developed, including dozens of agents that have ultimately received regulatory approval for use in patients[1]. These agents (which comprise small molecules and monoclonal antibodies), as well as hitherto experimental drugs targeting PI3K signalling at one of its nodes, are being (or already have been) extensively tested in combination with radiotherapy in hundreds of clinical trials.

The EGFR-targeting antibody cetuximab has been shown to improve the extent of locoregional tumour control and lead to extended OS in patients with HNSCC receiving definitive radiotherapy[216,217]. However, this approach is rarely used in clinical practice, as subsequent studies demonstrated the superiority of cisplatin-based chemoradiotherapy[218-220]. Moreover, the combination of cetuximab and radiotherapy has been associated with severe dermatitis (grade 3–4 in 32.5% of patients)[221], reflecting the convergence of toxicities separately associated with each approach[222]. The EGFR inhibitors gefitinib and erlotinib also improve response rates in patients with brain-metastatic NSCLC receiving whole-brain radiotherapy[223], as do bevacizumab and cediranib in patients with recurrent high-grade glioma[224] and newly diagnosed glioblastoma[225,226], respectively. Moreover, data from a pilot study suggest that <250-mg daily doses of apatinib can be safely combined with palliative radiotherapy in men with metastatic prostate cancer, resulting in synergistic effects on pain management[227].

Promising findings on safety and clinical activity have been obtained by combining radiotherapy with the experimental EGFR-targeting antibody nimotuzumab in patients with various solid tumours including NHSCC[228], glioblastoma[229] and diffuse intrinsic pontine glioma[230]. In a small pilot trial involving 11 patients with stage III–IVB HNSCC, alpelisib combined with cetuximab and intensity-modulated radiotherapy was well tolerated and was associated with a radiological complete response in all patients[231]. Similarly, daily alpelisib (200 mg) appears to be a safe combination partner for concurrent cisplatin-based chemoradiation in patients with locoregionally advanced HNSCC, with dose-limiting toxicities emerging only at the 250-mg dose[232]. However, data on the clinical efficacy of this combination are currently not available. Buparlisib has been tested in combination with palliative thoracic radiotherapy (20 Gy in five fractions) in a cohort of 22 patients with NSCLC, demonstrating an acceptable safety profile, target engagement and a reduction in tumour hypoxia[233]. Conversely, the unacceptable toxicities of buparlisib and radiotherapy combined with temozolomide documented in patients with newly diagnosed glioblastoma led to discontinuation of this approach[234]. Similarly, both pictilisib and dactolisib have been discontinued owing to limited therapeutic efficacy and a high risk of gastrointestinal toxicities[235-239].

Daily everolimus (an MTOR inhibitor) appears to be safe and tolerable in combination with fractionated radiotherapy following prostatectomy in men with prostate cancer[240], as well

as combined with chemoradiation in patients with locally advanced rectal cancer[241]. Again, results in patients with newly diagnosed glioblastoma were disappointing. Specifically, adding everolimus to radiotherapy plus concurrent and adjuvant temozolomide failed to improve progression-free survival (PFS) despite an increase in toxicity in a phase II study[242]. On the contrary, several randomized controlled trials have demonstrated that trastuzumab following adjuvant radiotherapy improves locoregional control and prolongs both PFS and OS in patients with $HER2^+$ breast cancer[243-247], albeit with a risk of sporadic acute cardiotoxicities[248,249]. Similarly, combining radiotherapy with lapatinib in patients with $HER2^+$ breast cancer[250] or HNSCC[251,252] appears to be safe, with at least some clinical activity. Conversely, the clinical development of the MET inhibitor JNJ38877605 has been abandoned owing to excessive renal toxicities[253]. Nonetheless, approaches combining radiotherapy with JNJ38877605, dactolisib or other agents that are poorly tolerated as monotherapies might be feasible owing to the potential for lower (and hence less toxic) doses of these agents to be used, potentially opening avenues for further clinical investigation.

Official sources list hundreds of ongoing clinical studies investigating the use of radiotherapy in combination with FDA-approved (and less so investigational) RTK and/or MTOR inhibitors, generally involving concurrent administration schedules and enrolling patients for whom these drugs are approved as monotherapies. Conversely, only a few studies assessing the therapeutic profile of radiotherapy combined with PI3K inhibitors are currently ongoing and these largely focus on patients with primary or metastatic brain lesions (NCT03696355, NCT04192981) and HNSCC (NCT02113878), two indications in which patients usually receive radiotherapy as part of the current SOC.

TGFβ signalling

TGFβ antagonizes the cytotoxic effects of radiotherapy by promoting DNA repair and hence favouring cancer cell survival (BOX 3). Accordingly, TGFβ has attracted attention as a promising target of novel anticancer therapeutics, leading to the development of various agents targeting canonical TGFβ signalling, including the TGFβ-targeting antibody fresolizumab as well as inhibitors of transforming growth factor-β receptor 1 (TGFβR1) or the TGFβ effectors smooth muscle and MAD-related protein 2 (SMAD2) and SMAD3, some of which are currently being tested in patients as monotherapies or in combination with radiotherapy[254] (Supplementary Table 3).

Genetic and pharmacological inhibition of TGFβ1 signalling in mammary epithelial cells leads to compromised ATM phosphorylation and ultimately to improved radiosensitivity[255]. Moreover, loss of TGFβ competence in HPV^+ HNSCC cells results in a defective DDR and increased sensitivity to DNA-damaging agents, including radiation[256]. Similarly, pretreatment with the TGFβ-neutralizing antibodies 13C4 and 1D11, as well as the TGFβR1 inhibitor LY364947, aggravates the loss of clonogenicity imposed by irradiation on cultured human breast cancer cells, and enhances the control of mouse 4T1 mammary tumours established in syngeneic immunocompetent mice achieved by single-fraction (8 Gy) and fractionated (12 Gy in three fractions) radiotherapy[257]. Comparable findings have been obtained in preclinical models of glioblastoma[258-260] and NSCLC[261]. TGFβ also appears

to promote the maintenance of the stem cell pool (which is enriched in the setting of acquired radioresistance) in multiple cancers[262]. Accordingly, treatment with the dual TGFβR1/TGFβR2 inhibitor LY2109761 has been associated with attenuation of radiation-driven epithelial-to-mesenchymal transition in a preclinical model of glioblastoma involving the orthotopic implantation of stem-like glioblastoma precursor cells in immunodeficient mice[259].

Notably, radiotherapy-elicited TGFβ signalling also promotes systemic effects outside the radiation field, including accrued tumour dissemination as a consequence of metastatic niche formation and inhibition of antitumour immunity. For example, thoracic radiation (10 Gy) elevates circulating TGFβ1 levels in mice bearing MMTV/PyVmT-driven mammary carcinomas, which correlate with increased numbers of blood-borne malignant cells and aggravated metastatic seeding[263]. In line with this notion, inhibition of autocrine or paracrine TGFβ signalling with the neutralizing antibody 2G7, as well as conditional deletion of *TGFBR2,* reduces the extent of radiation-initiated metastatic dissemination in the MMTV/PyVmT model[263]. TGFβ also has robust immunosuppressive effects that antagonize the ability of fractionated irradiation to elicit anticancer immunity[264]. Thus, administration of 1D11 enables the regression of syngeneic mammary and colorectal tumours exposed to fractionated radiation as it elicits systemic antitumour immunity against non-irradiated lung metastases or synchronous tumours (the so-called abscopal effect)[264,265]. This effect correlates with reduced phosphorylation of SMAD2 and SMAD3, as well as a genetic signature of IFNγ signalling with compensatory upregulation of PD-L1 and PD-L2 (REFS[264,265]). Accordingly, the addition of an anti-PD-1 antibody has been shown to extend the survival benefits enabled by radiation plus TGFβ blockade in several preclinical tumour models[264-266]. Moreover, dual inhibition of TGFβR2 and PD-L1 using the bifunctional antibody bintrafusp-α has been shown to strongly enhance the extent of radiotherapy-induced antitumour immunity compared with interventions targeting either pathway alone[267,268].

Importantly, TGFβ also mediates fibrotic reactions and promotes injury to non-malignant tissues, hence contributing to the adverse effects of radiotherapy on certain organs. In a cohort of patients with NSCLC receiving definitive radiotherapy, the development of radiotherapy-induced lung injury and poor clinical responses were associated with high circulating TGFβ levels[269]. Mice exposed to focal radiation can develop pulmonary fibrosis[270], oral mucositis[271] and skin irritation[272] correlating with accrued TGFβ signalling and SMAD activation. Moreover, pharmacological induction of TGFβ1 exacerbates radiation-induced heart and intestinal injuries in rats[273]. Consistent with this notion, LY2109761 has been shown to limit the extent of SMAD1 and SMAD2 phosphorylation, as well as the radiation-induced expression of genes associated with inflammation or angiogenesis, such as *Il7* in the irradiated mouse lung, ultimately having an antifibrotic effect[274]. Similarly, topical administration of recombinant human SMAD7 (which represses the activation of SMAD proteins involved in TGFβ signalling)[271] fused with a cell-penetrating peptide, alleviated radiotherapy-induced oral mucositis in an orthotopic xenograft model of oral cancer[275], while genetic inhibition of SMAD3 via transcutaneous delivery of a specific small-interfering RNA ameliorated skin irritation after high-dose irradiation (45 Gy) in mice[272]. Altogether, these findings strongly support the therapeutic

targeting of TGFβ signalling in order to inhibit the cytoprotective, immunosuppressive and profibrotic pathways elicited by radiotherapy.

Clinical considerations.

No specific TGFβ-targeted therapies are currently approved for use in patients with cancer[276]. The combination of fresolimumab (a fully human antibody directed against human TGFβ1, TGFβ2 and TGFβ3) plus radiotherapy (7.5 Gy in three fractions) has been evaluated in a randomized phase II trial involving women with previously treated metastatic breast cancer[277]. This trial was designed to compare two different doses of fresolimumab (1 mg/kg versus 10 mg/kg) with targeted radiotherapy delivered to a single metastatic lesion. Toxicities were deemed acceptable (only two of 23 treated patients developed keratoacanthoma) although clinical responses were limited to stable disease in three patients with no abscopal responses demonstrated[277], potentially owing to immune dysfunction at baseline[278]. Nonetheless, patients receiving 10 mg/kg fresolimumab had significantly longer median OS durations than those receiving the 1-mg/kg dose (16.0 months versus 7.6 months; HR 2.73, 95% CI 1.02–7.30; $P = 0.039$)[277]. Investigations of immune parameters demonstrated better activation of anticancer immunity in patients receiving the 10-mg/kg dose, manifesting as an increase in peripheral blood mononuclear cell counts and an enhanced $CD8^+$ T cell central memory pool[277]. Potentially explaining the limited objective response rate observed in this trial, radiation combined with inhibition of TGFβ signalling has been shown to drive the secretion of inhibin subunit-β A (INHBA) homodimers from mouse and human breast cancer cells, which are known to support the recruitment of immunosuppressive regulatory T cells in vivo and might explain the lack of responsiveness among patients receiving fresolimumab[279]. A randomized phase I/II study investigating the benefit of adding galunisertib (a selective TGFβR1 inhibitor) to temozolomide-based chemoradiotherapy in patients with newly diagnosed glioma revealed no differences in efficacy, safety or pharmacokinetic variables between the two treatment arms[280]. Thus, although safety appears to be acceptable, regimens combining radiotherapy with inhibition of TGFβ signalling might require the addition of other immunomodulatory agents to achieve clinical efficacy[281]. A number of ongoing clinical trials are currently investigating this approach in a variety of indications including early-stage NSCLC (NCT02581787), metastatic breast cancer (NCT03524170, NCT04756505), intrahepatic cholangiocarcinoma (NCT04708067), oesophageal squamous cell carcinoma (NCT04481256) and nasopharyngeal carcinoma (NCT04605562), in some of these settings as part of a multimodal treatment regimen also including ICIs or ICI-like strategies. The results of these studies are eagerly awaited.

Autophagy

Reflecting its potent cytoprotective effects, autophagy (BOX 4) has attracted considerable attention as a target of compounds that can be utilized clinically as chemosensitizers or radiosensitizers[282]. However, a variety of hitherto unresolved challenges have prevented the identification of clinically viable inhibitors of autophagy other than chloroquine and hydroxychloroquine, two non-specific lysosomal fusion inhibitors that are mainly used for malaria prevention[283] (Supplementary Table 3).

Radiotherapy drives dose-dependent autophagic responses driven by DNA damage and ROS production coupled with endoplasmic reticulum (ER) stress[284,285], and several reports have proposed a link between increased autophagic flux and the cytotoxicity of radiotherapy. Most of these studies, however, harnessed non-specific intervention strategies such as MTOR inhibitors (which are known to have radiosensitizing effects owing to inhibition of PI3K signalling, as discussed above)[286-290] or calorie restriction (which operates at the whole-body level)[291,292] to promote autophagy. Moreover, these studies have often drawn conclusions based on knockdown of a single component of the apparatus, despite virtually all of them having a plethora of concurrent autophagy-independent functions[293,294]. Conversely, proficient autophagic responses support the survival of irradiated cells, at least in part by antagonizing the generation of ROS[295] and promoting DNA repair via homologous recombination[296]. Indeed, autophagy defects typically result in the downregulation of proteins involved in homologous recombination (such as CHEK1) and other DNA repair pathways[296,297], as well as inhibition of DDR-related histone ubiquitination upon accumulation of the autophagic substrate sequestosome 1 (SQSTM1, best known as p62)[298], culminating in exacerbated accumulation of radiotherapy-elicited DSBs and global genomic instability[299]. Moreover, preclinical data suggest that hypoxia-associated radioresistance might, at least partially, involve activation of autophagy[300,301].

High levels of autophagy have been linked with radioresistance in HNSCC cell lines[302] and in patients with CRC[303]. Moreover, chloroquine has been shown to enhance the ER-stress-linked death of cultured mouse sarcoma cells driven by radiation[304] as well as the radiosensitivity of mouse breast cancer cells growing in immunocompetent syngeneic mice[58]. Similar effects have been observed with 3-methyladenine, a non-specific inhibitor of the autophagy-related PI3K catalytic subunit type 3, in preclinical models of HCC and in oesophageal cancer xenografts[305,306]. These findings are supported by the radiosensitizing effects of genetic methods of autophagy inhibition, including the miR-214-dependent silencing of ATG12 (REF.[303]) in cultured CRC cells, as well as the deletion of *Atg5, Atg7* or beclin 1 (*Becn1*) in mouse models of CRC and breast cancer, both in vitro and in vivo[58,307]. Consistent with this notion, proficient autophagic responses protect the non-malignant bone marrow following irradiation[308], largely by compensating for radiation-induced genotoxic stress via BRCA1-dependent DDR activation[309]. Of note, depletion of BECN1 results in a compromised radiation-induced DDR independent of autophagy[310], lending further support to the notion that signalling pathways not directly involved in autophagy that are nonetheless controlled by components of the autophagic machinery might also have a role in the acquisition of radioresistance[294].

Importantly, autophagy is also involved in the regulation of both natural and radiotherapy-driven anticancer immunity in a highly context-dependent manner[311-313]. Indeed, stable depletion of ATG5 or BECN1 compromises the sensitivity of syngeneic immunocompetent mouse models of CRC exposed to single-dose irradiation (8 Gy)[307]. Conversely, the deletion of *Atg5* or *Atg7* ameliorates the efficacy of fractionated irradiation (8 Gy in three fractions) in mouse mammary carcinomas growing in syngeneic immunocompetent hosts[58]. Data from mechanistic experiments demonstrate that this apparent discrepancy reflects the fact that anticancer immunity driven by these models preferentially involves autophagy-dependent ATP release versus autophagy-inhibited type I IFN secretion, respectively[58,307]. Whether

such a different requirement for ATP release versus type I IFN secretion depends on radiation dose, cell type or other unknown variables remains to be determined.

Clinical considerations.

Chloroquine and hydroxychloroquine are both available as potential combination partners for radiotherapy (and potentially other treatments including chemotherapy) in clinical settings[314], although the safety profile of these agents is far from optimal, probably reflecting their broad lysosomotropism[315]. Consistent with this notion, a phase I–II clinical trial testing hydroxychloroquine plus radiotherapy and temozolomide in patients with newly diagnosed glioblastoma identified a maximum tolerated dose of 600 mg/kg per day, a dose that was insufficient to achieve consistent inhibition of autophagy in patients and failed to improve OS[316]. Along similar lines, a single-centre, open-label, dose-finding phase I trial enrolling patients with newly diagnosed glioblastoma identified 200 mg/day as the maximum tolerated dose for chloroquine when administered in combination with radiotherapy (59.4 Gy in 33 fractions), resulting in severe toxicities (six chloroquine-related clinically serious events and one death) and a median OS duration of 16 months (which is comparable to that achieved with SOC approaches)[317]. Conversely, 150 mg/day chloroquine combined with whole-brain irradiation (30 Gy in ten fractions over 2 weeks) did not affect quality of life outcomes or increase the incidence or severity of adverse events in patients with brain metastases enrolled in a randomized double-blind, placebo-controlled phase II study[318]. However, despite a mild amelioration of brain metastases-specific PFS (relative risk 0.31, 95% CI 0.1–0.9; $P = 0.046$), no significant difference in OS could be documented relative to radiotherapy alone[318]. Similar findings emerged from a pilot study involving 20 patients with newly diagnosed brain metastases from primary lung, breast or ovarian cancers who received daily chloroquine (250 mg) and whole-brain radiotherapy (37.5 Gy in 2.5-Gy daily fractions)[319]. Accordingly, very few clinical trials testing chloroquine or hydroxychloroquine remain active. Trials continuing to investigate this approach include a phase II study testing radiotherapy plus hydroxychloroquine and capecitabine in patients with resectable pancreatic cancer (NCT01494155), a phase I trial investigating partial brain radiotherapy plus temozolomide, chloroquine and tumour-treating fields in patients with newly diagnosed glioblastoma (NCT04397679), and a phase II study assessing the effects of radiotherapy combined with chloroquine in the same indication (NCT02432417). Thus, although an abundant preclinical literature suggests that inhibition of autophagy stands out as a promising strategy to improve the efficacy of radiotherapy, the clinical translation of this concept is hindered by the lack of safe and specific inhibitors[283]. Further complicating this issue, strategies involving the specific delivery of autophagy inhibitors to cancer cells might have to be developed for these agents to achieve acceptable tolerability and good efficacy (alone as well as combined with radiotherapy), thus reflecting the key role of autophagy in the initiation of tumour-targeting immunity[320].

Conclusions

Taken together, the data discussed herein lend strong support to the notion that radiotherapy can be used to activate cytoprotective signalling pathways associated with radioresistance and thus create a state of non-oncogene addiction that renders tumour cells vulnerable to

targeted therapies (FIG. 2). With only a few exceptions, however, this concept has yet to be implemented in the clinic, reflecting the existence of several obstacles in translating results from preclinical settings. Nonetheless, a few key points emerge from the literature discussed above.

Firstly, administration schedule clearly has a critical role in the efficacy of therapeutic regimens involving radiotherapy[116,196,215]. However, only a few preclinical studies have thus far compared the efficacy of different treatment schedules with the aim of identifying an optimal approach prior to focusing on mechanistic aspects. Thus, different treatment schedules might have resulted in superior preclinical and possibly clinical efficacy in at least some of the studies that have suggested only limited synergy between radiotherapy and targeted therapies.

Secondly, clinical studies investigating the role of radiotherapy in combination with agents targeting the mechanisms discussed herein have typically used conventional fractionation schedules. However, an expanding body of literature demonstrates that both dose and fractionation schedule have clinically relevant effects on the signal transduction cascades elicited by radiotherapy. For example, radiation delivered as three fractions of 8 Gy each has robust immunostimulatory effects in preclinical models of breast cancer, while a single radiation dose of 20 Gy fails to do so as a consequence of the upregulation of a cytosolic exonuclease (TREX1) that shuts down type I IFN secretion by irradiated cells[321]. This observation implies that combining radiotherapy with hitherto experimental TREX1 inhibitors would result in limited synergy if the radiation dose and fractionation schedules employed failed to elicit meaningful TREX1 expression. Similar considerations apply to each of the pathways discussed herein, for which limited preclinical investigation of optimal dose and fractionation approaches has been undertaken prior to clinical testing. As an added layer of complexity, recapitulating the standard radiotherapy regimens used clinically (such as 2 Gy in 30 fractions, which is commonly used for the clinical management of patients with NSCLC)[322] is not always feasible in preclinical models. At least in part, this lack of accurate modelling reflects the practical, ethical and experimental constraints associated with delivering anaesthesia to rodents on a daily basis over several weeks[323].

Thirdly, most of the preclinical studies testing radiation in combination with agents targeting radiotherapy-driven non-oncogene addiction have involved human cancer cell lines maintained in vitro or xenografted into immunodeficient hosts. While cell lines offer a number of advantages, novel experimental platforms including patient-derived organoids and patient-derived xenografts might be superior in terms of recapitulating neoplasm-specific features and enabling the identification of personalized combination regimens that might be highly effective (although difficult to test in large patient cohorts)[324,325]. That said, both patient-derived organoid and patient-derived xenograft models also fall short in assessing the potential effects of treatment on the immune system (be it positive, and hence supporting efficacy, or detrimental, and hence limiting efficacy). Indeed, both radiotherapy[16,326] and targeted anticancer agents[93] are now known to mediate a number of clinically relevant immunomodulatory effects that can no longer be overlooked in an era in which translational research is increasingly being used to guide the design of clinical studies.

Finally, clinically actionable biomarkers enabling the identification of tumours that are likely to respond to radiotherapy in combination with drugs that block the cytoprotective signalling pathways associated with radioresistance are generally missing[327]. In specific settings (such as radiotherapy combined with PARP inhibitors), baseline features that are associated with treatment sensitivity might exist (such as homologous recombination defects), but remain difficult to assess in a reliable manner[89]. In other scenarios, for example regimens combining radiotherapy with autophagy inhibitors, activation of autophagy might emerge only after irradiation, which renders assessment even more complex (at least within the tumour microenvironment). Whether circulating biomarkers can be helpful in this setting remains completely unexplored.

Of note, growing levels of expectation exist regarding the efficacy of regimens that combine radiotherapy with anti-PD-1 or anti-PD-L1 antibodies in patients with various cancer types, reflecting encouraging data from clinical trials involving patients with advanced-stage NSCLC[29,328,329]. Intriguingly, PD-L1 expression at baseline has been associated with improved responsiveness to ICIs in multiple clinical settings[330], although patients with NSCLC appear to obtain benefit from the addition of durvalumab to chemoradiotherapy irrespective of baseline PD-L1 status[331]. Despite a lack of mechanistic evidence, it is tempting to speculate that such an observation reflects the ability of radiotherapy to promote PD-L1 expression via several different mechanisms, as discussed in this Review. Further supporting this possibility, patients with PD-L1^{-} NSCLC at baseline appear to obtain greater levels of benefit from the addition of SBRT to pembrolizumab than those with PD-L1^{+} NSCLC (in whom pembrolizumab is more likely to be active as monotherapy)[332]. Yet another promising strategy involves combining radiotherapy with agents that target inhibitor of apoptosis proteins (IAPs), such as xevinapant[333,334]. Whether radiotherapy is capable of driving the upregulation or activation of IAPs, however, remains unclear.

In conclusion, while additional research is needed, we surmise that well-designed preclinical studies conceived to comparatively assess various dose and fractionation schedules in immunocompetent preclinical models could unlock the clinical potential of radiotherapy as a means to elicit cytoprotective pathways that can then be inhibited using targeted anticancer therapies. Future studies will reveal which patient subgroups can derive superior clinical benefits from this innovative use of radiotherapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The work of L.C.C. is supported by US National Institutes of Health (NIH) P01 (#P01CA120964) and R35 (#R35CA197588) grants. The work of S.C.F. is supported by a Breakthrough Level 2 grant from the US Department of Defense (DoD), Breast Cancer Research Program (BRCP) (#BC180476) and by the 2019 Laura Ziskin Prize in Translational Research (#ZP-6177) from Stand Up to Cancer (SU2C). The work of L.G. is supported by a Breakthrough Level 2 grant from the US DoD BRCP (#BC180476P1), by the 2019 Laura Ziskin Prize in Translational Research (#ZP-6177, PI: Formenti) from SU2C, by a Mantle Cell Lymphoma Research Initiative (MCL-RI, PI: Chen-Kiang) grant from the Leukaemia and Lymphoma Society (LLS), by a startup grant from the Department of Radiation Oncology at Weill Cornell Medicine (New York, USA), by a Rapid Response Grant from the Functional Genomics Initiative (New York, USA), by industrial collaborations with Lytix (Oslo, Norway) and

Phosplatin (New York, USA), and by donations from Phosplatin (New York, USA), the Luke Heller TECPR2 Foundation (Boston, USA), Onxeo (Paris, France), Ricerchiamo (Brescia, Italy) and Sotio a.s. (Prague, Czech Republic).

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Box 1 |

Cytotoxic pathways elicited by radiotherapy

Radiotherapy mediates cytotoxic effects that originate either from direct damage or reactive oxygen species (ROS)-dependent damage to macromolecules. According to a commonly accepted model, DNA is the macromolecule most affected by radiotherapy, resulting in a variety of lesions with a predominance of double-stand breaks. These lesions rapidly activate the so-called DNA damage response (DDR), which initially operates in an adaptive, cytoprotective mode, involving a temporary arrest of cellular proliferation (generally at the G2–M transition) that enables DNA repair and the recovery of cellular homeostasis. However, if DNA damage is excessive and ultimately remains unrepaired, the DDR can switch to a cytotoxic mode, in which it can initiate the active demise of cells with DNA damage deemed to be beyond repair[335]. Such a cytotoxic DDR most often involves activation of p53, which upon phosphorylation by ATM or the ATM substrate CHEK2 is stabilized and coordinates the expression of various proteins involved in mitochondrial apoptosis, including BAX and its activators BBC3 (also known as PUMA) and PMAIP1 (also known as NOXA). PUMA, NOXA and other so-called BH3 only proteins favour the oligomerization of BAX and BAK1 at the outer mitochondrial membrane, culminating in its permeabilization, and (1) irreversible mitochondrial inactivation coupled with oxidative macromolecular damage (the actual cause of cell death) and (2) the activation of proteolytic enzymes including caspases (which regulate the kinetic and immunological manifestations of cell death)[13,266]. Importantly, especially in the context of p53 defects, cancer cells arrested by radiotherapy at the G2–M transition can illicitly slip into defective mitosis characterized by multinucleation or micronucleation[336]. This process, which is commonly referred to as mitotic catastrophe, ultimately leads to cell death (either during mitosis, or during interphase in daughter cells) or permanent proliferative inactivation (so-called cellular senescence) resulting in mitosis-incompetent cells[336]. Of note, data published in 2020 point to extranuclear damage, especially oxidative damage to lipid layers, as another contributor to the anticancer effects of radiotherapy[337,338]. In this setting, cytotoxicity emerges from a non-apoptotic variant of regulated cell death commonly known as ferroptosis[337,338]. Intriguingly, ROS participate in both DDR-dependent apoptosis and ferroptosis, which is in line with an abundant literature linking hypoxia with radioresistance[24].

Box 2 |

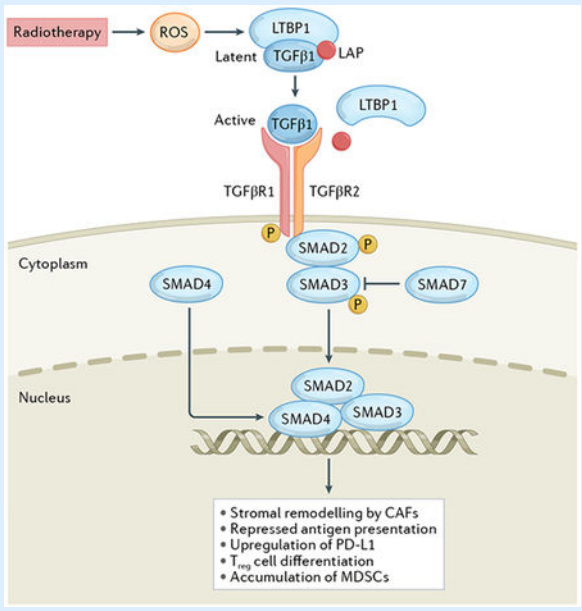
Immunomodulatory pathways elicited by radiotherapy

Besides promoting senescence and the death of malignant cells, radiotherapy mediates a panel of immunostimulatory effects that (at least in certain settings) are expected to contribute to clinical efficacy. These effects largely reflect the ability of radiotherapy to promote the antigenicity and adjuvanticity of cancer cells downstream of: (1) transcriptional upregulation of genes that encode antigenic neoepitopes that are otherwise silenced, resulting in the engagement of adaptive immunity[339]; (2) increased exposure of MHC class I molecules on the cell surface, thus facilitating the recognition of irradiated cells by $CD8^+$ T cells[340]; (3) upregulation of natural killer cell activating ligands, thus favouring the activation of antigen-independent effector responses[340]; (4) accumulation of mitochondrial DNA in the cytosol of irradiated cells, culminating in type I interferon secretion upon activation of cyclic GMP–AMP synthase[58,341]; and (5) activation of immunogenic cell death and the consequent release of multiple immunostimulatory molecules that ultimately support T cell activation, including a panel of so-called damage-associated molecular patterns[342]. However, radiotherapy can also mediate immunosuppressive effects, including the upregulation of PD-L1[340] and the accumulation of $CD4^+CD25^+FOXP3^+$ regulatory T cells[279]. Thus, at least in immunologically competent tumours (those that are susceptible to anticancer immunity), the efficacy of radiotherapy is influenced by the balance between activation of immunostimulatory and immunosuppressive signalling pathways. Importantly, many of these pathways can be harnessed using immunotherapeutic approaches designed to enhance the efficacy of radiotherapy, as currently investigated in a large number of clinical trials[343].

Box 3 |

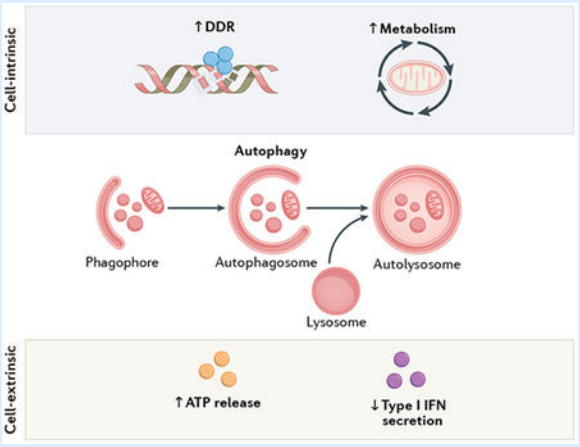
TGFβ signalling

TGFβ is a pleiotropic cytokine with a wide range of biological effects on a number of targets, including (but not limited to) malignant, stromal and immune cells[254]. Although TGFβ is generally regarded as an tumour-suppressive agent, it can paradoxically also support tumour progression via several cancer cell-intrinsic mechanisms, including the epithelial-to-mesenchymal transition and the acquisition of stem-like features[254]. Moreover, TGFβ production by malignant cells and cancer-associated fibroblasts (CAFs) promotes a variety of immunosuppressive effects, including repressed antigen presentation on MHC class I and the upregulation of PD-L1 by cancer cells, as well as the stimulation of regulatory T cell differentiation and tumour infiltration by myeloid-derived suppressor cells (MDSCs)[254,344]. TGFβ is also involved in stromal remodelling driven by CAFs, which favours the establishment of a fibrotic tumour microenvironment that arrests infiltration by tumour-targeting T cells and thus limits the efficacy of several therapies[345-347]. Activation of TGFβ in the irradiated tumour microenvironment depends on a series of highly regulated events beyond transcriptional upregulation: (1) assembly of a supramolecular complex containing a long TGFβ precursor and latent TGFβ binding protein 1 (LTBP1), which occurs in the endoplasmic reticulum; (2) proteolytic and oxidative processing to obtain mature TGFβ complexed with the so-called latency-associated peptide (LAP) and LTBP1, which occurs in the Golgi apparatus; (3) secretion of the TGFβ–LAP–LTBP1 complex into the microenvironment; and (4) release of bioactive TGFβ[348,349]. On release from LAP, TGFβ binds to a heterodimeric receptor composed of TGFβ receptor 1 (TGFβR1) and TGFβR2, generally culminating in the activation of cytoprotective and immunosuppressive transcriptional programmes orchestrated by smooth muscle and MAD-related protein (SMAD) 2 (REF.[349]). Moreover, TGFβ signalling has also been linked to radioresistance as a consequence of robust DNA repair via ATM and p53 activation[350].



Box 4 | Autophagy

Autophagy is a catabolic process through which cytoplasmic material, including damaged or dispensable organelles and/or portions thereof, are sequestered within newly formed double-membraned vacuoles (known as autophagosomes) and delivered to lysosomes for degradation[351]. As such, autophagy generally mediates robust cytoprotective functions by supporting the preservation of cellular homeostasis in response to stress[351]. Indeed, the autophagic flux of material can be finely tuned in response to cellular demands[22]. Specifically, autophagy is mediated by members of the ATG family of evolutionarily conserved proteins, the coordinated activity of which is under tonic inhibition by MTOR[22]. Importantly, although proficient autophagy in non-malignant cells generally antagonizes malignant transformation by favouring the preservation of genetic, oxidative and metabolic homeostasis, established cancer cells can also harness autophagy in support of disease progression and resistance to therapy[352]. Moreover, autophagy has a context-dependent, dual role in the initiation of anticancer immunity, being necessary for the emission of danger signals by malignant cells responding to immunogenic chemotherapy, but also inhibiting both antigen presentation and radiotherapy-induced secretion of type I interferon (IFN)[353]. DDR, DNA damage response.



Key points

- Targeted anticancer agents are commonly used in the treatment of various solid and haematological malignancies.
- Not all tumours are sensitive to these agents, largely reflecting the lack of or inactivity of the targetable alteration.
- Radiotherapy is also frequently used for the treatment of cancer, owing to its prominent cytostatic and cytotoxic effects on malignant cells.
- A wide panel of cytoprotective pathways can be activated by radiotherapy, thus limiting therapeutic efficacy.
- However, these signal transduction cascades can be effectively inhibited with targeted anticancer agents, potentially supporting superior treatment efficacy.
- Radiotherapy stands out as a promising tool to elicit clinically actionable signalling pathways in cancer.

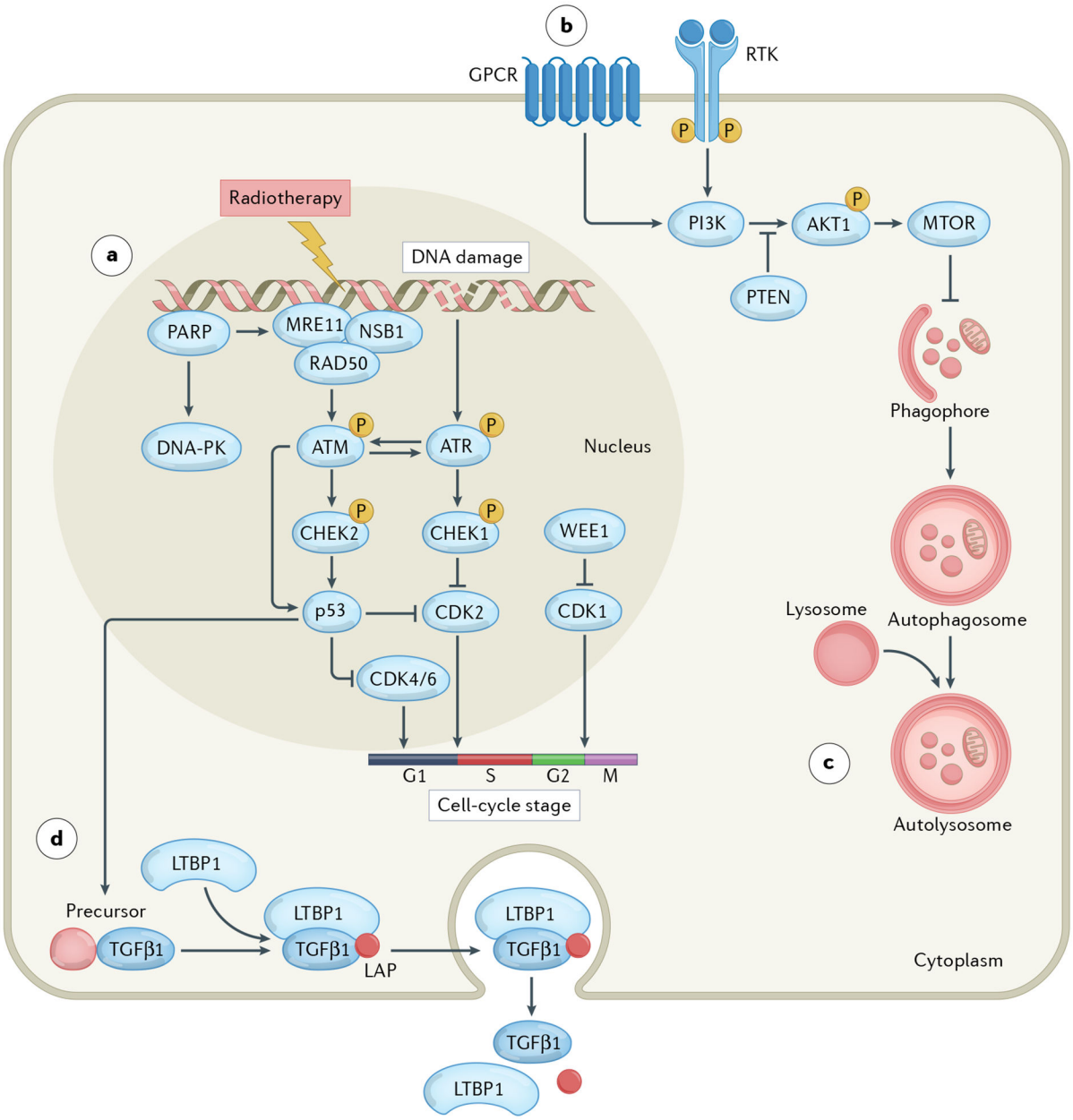


Fig. 1 |. Cytoprotective pathways elicited by radiotherapy.

Ionizing radiation damages a variety of macromolecules including nuclear DNA, either directly or upon generation of reactive oxygen species. Such damage is often detected by a molecular complex encompassing meiotic recombination 11 (MRE11), DNA repair protein Rad50 (RAD50) and Nijmegen breakage syndrome 1 (NSB1) in co-operation with members of the poly(ADP-ribose) polymerase (PARP) protein family. Formation of this complex results in the sequential activation of ATM, CHEK2 and p53. Alternatively or concomitantly, the DNA damage induced by radiotherapy drives the activation of ATR and consequently CHEK1, DNA-dependent protein kinase (DNA-PK) or WEE1 signalling. Ultimately, these pathways converge on the inhibition of cyclin-dependent kinases (CDKs) resulting in arrested cell-cycle progression at specific checkpoints, which enables DNA

repair and hence supports radioresistance (part **a**). The DNA damage response elicited by radiotherapy also promotes (directly or indirectly) the hyperactivation of PI3K signalling, resulting in the delivery of cytoprotective signals via AKT1 and MTOR (part **b**), the activation of autophagy (which is generally under negative regulation by MTOR) (part **c**), as well as the synthesis, secretion and activation of transforming growth factor-β1 (TGFβ1) (part **d**). GPCR, G-protein-coupled receptor; LAP, latency associated peptide; LTBP1, latent transforming growth factor-β binding protein 1; PTEN, phosphatase and tensin homologue; RTK, receptor tyrosine kinase.

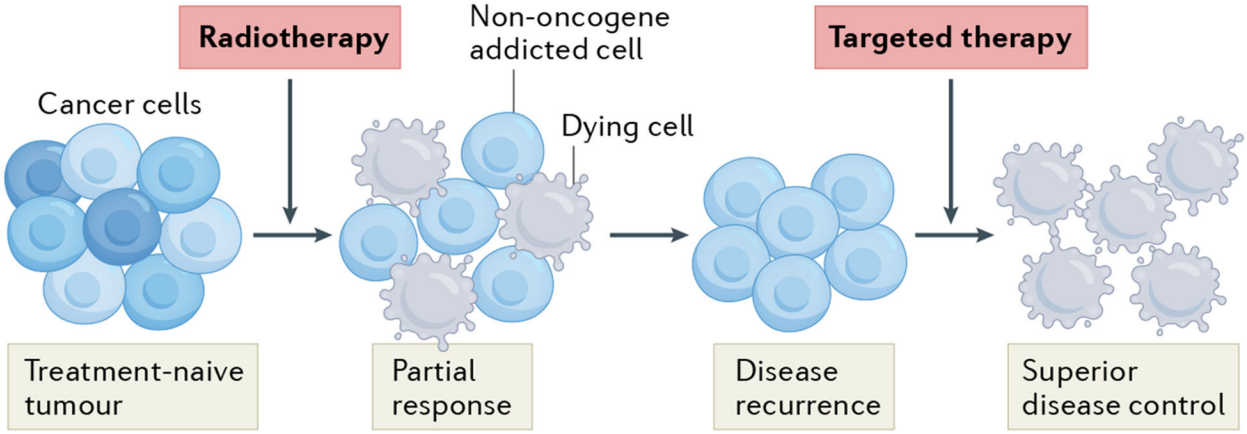


Fig. 2 |. Targeting the pro-survival pathways induced by radiotherapy in cancer.

At least in part owing to intratumoural heterogeneity, radiotherapy alone is often unable to kill all malignant cells and thus does not mediate complete tumour eradication. In this setting, cancer cells generally resist the cytostatic and/or cytotoxic effects of radiotherapy along with the activation of various cytoprotective signalling pathways. Importantly, such cytoprotective pathways establish a state of non-oncogene addition that can be targeted using specific agents in order to achieve superior disease control.