



Improving the Efficacy of Tumor Radiosensitization Through Combined Molecular Targeting

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Chemoradiation, either alone or in combination with surgery or induction chemotherapy, is the current standard of care for most locally advanced solid tumors. Though chemoradiation is usually performed at the maximum tolerated doses of both chemotherapy and radiation, current cure rates are not satisfactory for many tumor entities, since tumor heterogeneity and plasticity result in chemo- and radioresistance. Advances in the understanding of tumor biology, a rapidly growing number of molecular targeting agents and novel technologies enabling the in-depth characterization of individual tumors, have fuelled the hope of entering an era of precision oncology, where each tumor will be treated according to its individual characteristics and weaknesses. At present though, molecular targeting approaches in combination with radiotherapy or chemoradiation have not yet proven to be beneficial over standard chemoradiation treatment in the clinical setting. A promising approach to improve efficacy is the combined usage of two targeting agents in order to inhibit backup pathways or achieve a more complete pathway inhibition. Here we review preclinical attempts to utilize such dual targeting strategies for future tumor radiosensitization.

Keywords: radiotherapy, radioresistance, radiosensitization, combined molecular targeting, dual inhibition

INTRODUCTION

Chemoradiation is a current standard of care for the curative treatment of most locally advanced solid malignancies. Both modalities are generally administered at the maximum-tolerated doses to achieve best possible cure rates, which for many entities such as lung, brain, colorectal, bladder, or human Papillomavirus (HPV)-negative head and neck cancer, are still far from satisfactory. Due to the intense treatment regimes a considerable fraction of patients suffer from severe acute as well as late and partly irreversible side effects that can seriously impact quality of life. For example in head and neck squamous cell carcinoma (HNSCC) the addition of platin-based chemotherapy to radiotherapy increases 5-year overall survival by about 10% (1, 2) at the cost of increases in the rate of severe adverse events, such as grade 3 mucositis, anemia and nephro- and ototoxicity, which can result in lifetime renal insufficiency and hearing loss (3).

Combining radiotherapy with molecular targeting agents may offer an alternative to chemoradiation with potentially less severe side effects, provided the tumor cells are more dependent on the specific target than normal tissue. To be effective, the targeting agent needs to be directly toxic for the tumor and/or has to induce a meaningful radiosensitization. Despite a plethora of promising preclinical data, the results achieved in the clinic are so far exceedingly disappointing. The only currently approved molecular targeting agent for the combination with radiotherapy is the anti-epidermal growth factor receptor (EGFR)-antibody cetuximab in HNSCC. The combination was approved on the basis of the IMC 9815 phase III clinical trial, which demonstrated superiority over radiation alone in a range similar to the addition of cisplatin to radiotherapy (4). However, after a considerable number of subsequent publications it has to be seriously called into question whether the addition of cetuximab to radiotherapy is a viable alternative for cisplatin (5–7) and cetuximab also failed to enhance survival when added to chemoradiation (8). Recently, cetuximab-radiation was directly shown to be inferior to cisplatin-based chemoradiation in HPV-positive oropharyngeal cancer in two prospective phase III trials (9, 10) although this entity had shown the greatest benefit from cetuximab in the IMC 9815 trial (11).

A general limitation for the effective use of molecular targeted agents is the current lack of biomarkers that could predict a possible oncogenic addiction to a given druggable target or a possible role of the target in radiation resistance. Also in the case of cetuximab in HNSCC, no predictive biomarker has been established. In order to fully exploit the potential of precision medicine, such biomarkers are mandatory to select the best agents for a given tumor. Sequencing individual tumors for druggable driver mutations is one way forward. However, to what extent the targeting of such potential oncogenic driver proteins will also result in an enhanced sensitivity toward radiotherapy is currently unknown.

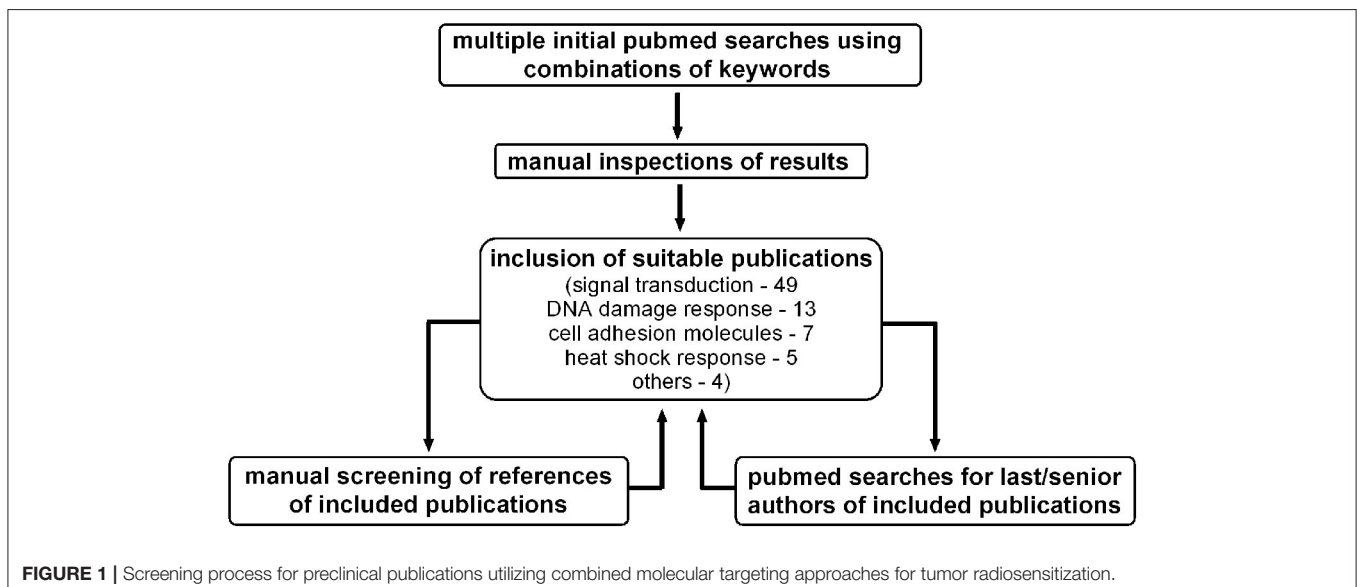
Another important concern is therapy resistance due to backup pathways or incomplete inhibition. In such cases, combined molecular targeting approaches may be an effective way to increase efficacy. Combined targeting often follows three main strategies: (1) blocking of potential alternative pathways, (2) dual targeting of the same pathway to achieve a more complete inhibition or (3) targeting of two distinct pathways whose dual inhibition will result in synthetic lethality or synergistic radiosensitization (12).

Here we review preclinical attempts to utilize such dual targeting strategies for future tumor radiosensitization.

METHODS

A PubMed search based on the key words “agent*, radiosensiti*, radiotherapy, molecular targeted therapy, combined molecular targeting” was conducted and the results were screened for use of combined molecular targeting for radiosensitization in the preclinical setting. In addition, because titles and abstracts do not follow any regular pattern, references from identified articles were further screened for suitable publications and PubMed was additionally screened for publications from the last/senior authors of identified articles (Figure 1).

Publications dealing with immunotherapy, e.g., using immune checkpoint inhibitors were not included, since they do not represent radiosensitization in the narrow sense. Publications of combined usage of molecular targeting and chemotherapy to achieve radiosensitization were also not included. Further, it was not always possible to discriminate between the intentional combined inhibition of two defined molecular targets and the less well-defined usage of somewhat unspecific agents with two or more targets. The latter were considered when reflecting the basic idea of the combined targeting approaches for radiosensitization, i.e., the intended selection of two targets whose inhibition should achieve at least additive or even synergistic effects.



Regarding clinical trials with published results, we performed a PubMed search with the respective targeting agents found in preclinical studies plus the terms “radiation” or “radiotherapy.” Since the focus of this review is on preclinical approaches, we only present a selection of the most important clinical trials.

RESULTS

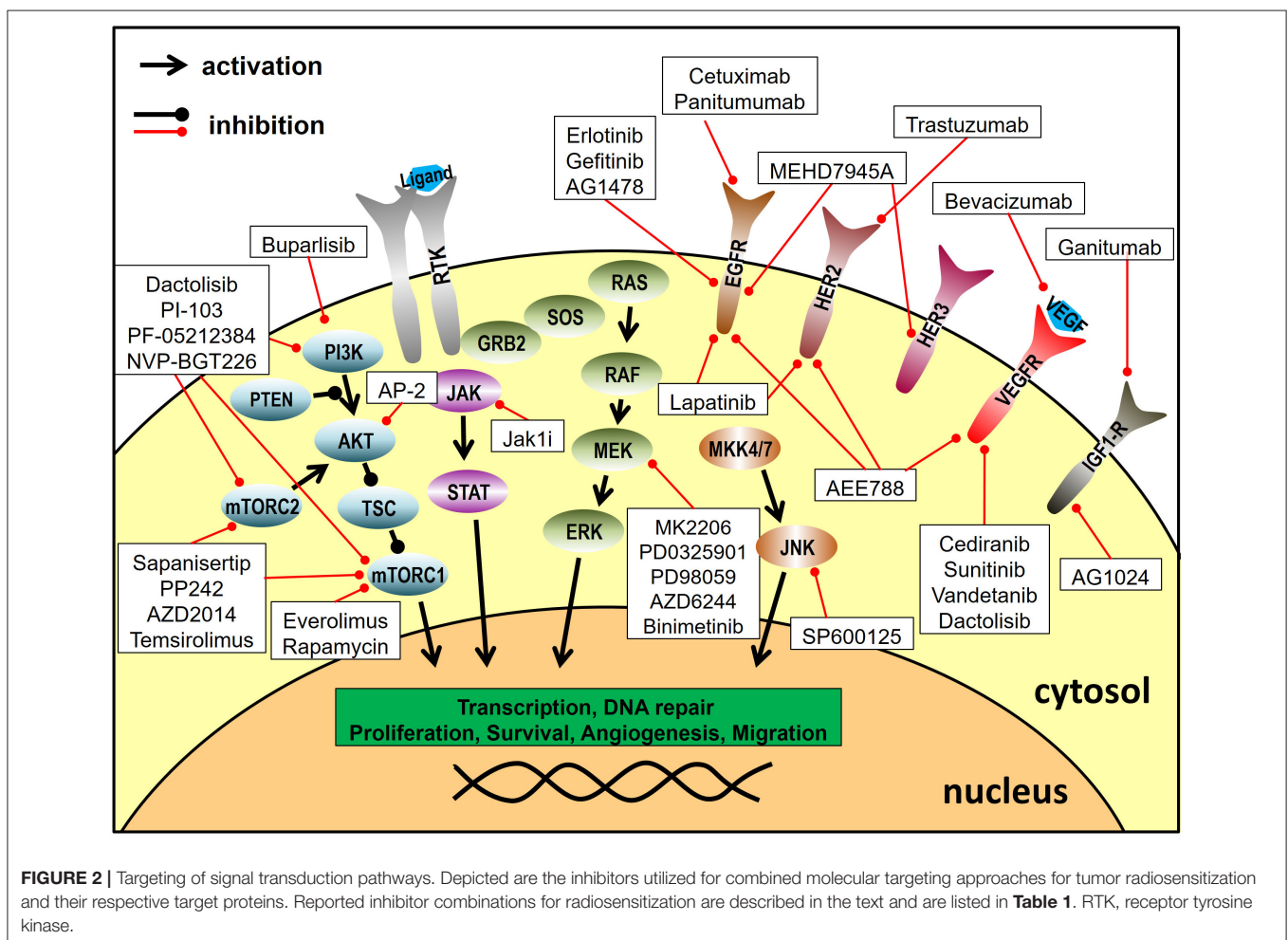
The vast majority of publications reporting experimental dual targeting approaches in combination with ionizing radiation fall into four categories: (1) growth factor receptor signaling, (2) DNA damage response and cell cycle checkpoints, (3) cell adhesion molecules, and (4) the heat shock response. From these categories targeting growth factor receptor signaling currently represents the by far most extensively studied dual targeting approach. In some of the identified papers inhibitors belonging to two of these categories were combined. These papers will only be presented in one section. Studies using a single substance with dual specificity were considered when its use was based on a rational selection of targets whose inhibition should achieve at least an additive or a synergistic effect.

Targeting Growth Factor Receptor Signaling

The most frequently used approach of radiosensitization through dual molecular targeting is the inhibition of growth factor receptor tyrosine kinases and their related signaling pathways. Growth factor receptor signaling can contribute to radioresistance, because it stimulates proliferation, inhibits apoptosis and has been described to increase the repair of radiation-induced DNA-damage, which makes it an attractive molecular target for radiosensitization (13, 14). Combined targeting approaches were further fuelled by the approval of the anti-EGFR monoclonal antibody (mAb) cetuximab in the curative treatment of HNSCC and by the desire to increase efficacy and repress by-pass signaling and resistance, which pose a potential risk to all signaling inhibition approaches (15). **Figure 2** provides an overview of the inhibited signaling pathways and proteins described in this section.

The HER Family

The HER sub-family of receptor tyrosine kinases (RTK) includes the members EGFR (also termed HER1 or ErbB1), Her2 (ErbB2),



HER3 (ErbB3), and HER4 (ErbB4). These transmembrane receptors are located at the cell surface, harbor an intrinsic protein kinase domain and regulate proliferation, migration, cell fate determination and apoptosis via diverse downstream signaling pathways such as MAPK and AKT signaling (16). EGFR is expressed in normal epithelial cells of the skin, hair follicles or the gastro intestinal tract, but it is also detected in many tumor entities. Furthermore, EGFR gene amplifications or mutations are found in e.g., HNSCC, lung cancer or glioblastoma (GBM), driving carcinogenesis and tumor progression (17, 18). Consequently, targeting EGFR with mAbs or tyrosine kinase inhibitors (TKI) has been established in cancer therapy in e.g. NSCLC, colorectal cancer, head and neck cancer, or pancreatic cancer but therapy resistance occurs frequently and compromises outcome (19). Usually, ligand-binding leads to ErbB receptor homodimerization but can also result in the formation of heterodimers consisting of different sub-family members. Due to these interactions and possible functional redundancies co-targeting of different sub-family members has been investigated in several pre-clinical studies.

HER/HER targeting

Combined inhibition of different members of the HER sub-family indeed showed promising results in terms of radiosensitization. For example in first studies Fukutome et al. combined the EGFR inhibitor gefitinib (TKI) and the anti-HER2 antibody trastuzumab. Both inhibitors induced radiosensitization on their own and their combination resulted in a synergistic sensitization in vulvar squamous cell carcinoma cells expressing EGFR and HER2 (20).

Also EGFR/HER-2 inhibition by the dual inhibitor lapatinib resulted in enhanced radiosensitivity in cancer cells of various entities, such as bladder cancer, peripheral nerve sheath tumors, pancreatic or breast cancer. This sensitization was shown to be partly dependent on the expression of the specific targets (HER2, EGFR) and to be inhibited through the constitutive activation of downstream signaling factors, such as Ras & Raf mutations (21–27).

To inhibit EGFR and HER3 Huang et al. used the dual inhibitor MEHD7945A. They demonstrated that MEHD7945A inhibits growth in cetuximab (EGFR mAb) and erlotinib (EGFR TKI) resistant cells with a significant PI3K and MAPK pathway inhibition. In a xenograft model, MEHD7945A reduced the growth of tumors resistant to mono-EGFR-targeting, and, in contrast to cetuximab, the combination with radiation resulted in a more pronounced growth inhibition than either modality alone. EGFR and HER3 are both activated upon radiation and the blockade of one receptor may be compensated by the other. Treatment with MEHD7945A but not with cetuximab reduced survival signaling and DNA repair (28). The same group could substantiate the evidence for a radiosensitizing effect of MEHD7945A using human lung and head and neck cancer cells as well as xenografts further supporting the clinical implementation of this EGFR/HER3 combined targeting approach (29).

HER/IGF-1R targeting

In addition to the formation of heterodimers within the HER-family there is also a cross talk between EGFR and other receptor tyrosine kinases such as the insulin like growth factor 1 receptor (IGF-1R), which is also involved in tumor development and progression (30). In this context Matsumoto et al. compared individual and dual targeting of EGFR and IGF-1R in an HNSCC xenograft model using the mAbs ganitumab (anti-IGF-1R) and panitumumab (anti-EGFR). They observed the strongest growth arrest and significantly fewer recurrences upon combined inhibition plus radiation (31). Wang et al. also showed a radiosensitizing effect of combined inhibition of EGFR through erlotinib and the IGF-1R inhibitor AG1024 in prostate cancer cells, suggesting a suppression of homologous recombination repair as a possible underlying mechanism (32). Using two breast cancer cell lines with similarly high expression of IGF-1R but differential expression of EGFR, Li et al. observed radiosensitization through IGF-1R-inhibition (AG1024) in both strains. The EGFR inhibitor AG1478, however, only radiosensitized the cell line with high EGFR-expression both alone and when added to IGF-1R-inhibition. Radiosensitization through combined targeting was further validated in a xenograft model (33).

HER/downstream targeting

The HER receptors transduce their signals through several downstream pathways including the Ras-Raf-MAPK, the PI3K-Akt and the JAK/STAT pathway (19, 34). Alterations within these pathways might affect the efficacy of HER inhibition as demonstrated by the importance of the Ras mutation status in colorectal cancer where patients carrying such mutations do not benefit from cetuximab treatment (35, 36). Therefore, another strategy to increase efficacy is to combine the inhibition of the receptors and relevant downstream targets.

In this context Bonner et al. assessed the effect of combined treatment of head and neck cancer cells with cetuximab and the JAK inhibitor JAK1i. STAT3 is a downstream protein activated by JAK (among others) protecting cells from apoptosis. The authors observed enhanced anti-proliferative and apoptotic effects upon dual inhibition plus radiation. Dual inhibition was accompanied by a more complete inhibition of STAT3-phosphorylation and, in contrast to single inhibition, resulted in radiosensitization in colony formation assays (37).

Eke et al. identified the activation of c-Jun N-terminal kinase 2 (JNK2) via the scaffold protein JNK-interacting protein 4 (JIP-4) as a possible signaling bypass after EGFR targeting. The authors knocked down JIP4 or JNK2 via siRNA and used the JNK2 inhibitor SP600125 in addition to cetuximab treatment and achieved enhanced tumor cell radiosensitization in an additive manner as compared to single inhibition (38).

Activation of the PI3K/Akt/mTOR pathway was demonstrated by Zhuang et al. in lung adenocarcinoma cells as another resistance mechanism against EGFR targeting. They could demonstrate that mTOR inhibition with everolimus enhanced radiation sensitivity when added to erlotinib *in vitro* and in a xenograft model (39).

HER/VEGF(R) targeting

The family of vascular endothelial growth factors (VEGFs) and their specific receptors (VEGFRs) are frequently targeted in cancer therapy, e.g., in lung, breast, kidney, ovarian and cervix cancer. A fundamental difference in this therapeutic strategy is that, although the inhibition of tumor cell signaling is also of relevance, the main target of VEGF(R)-inhibition is tumor angiogenesis. VEGFs and VEGFRs are critical factors in the formation and maintenance of new vasculature in both normal tissues and solid tumors (40, 41). Their inhibition can indeed follow two contrary intentions: (1) a complete inhibition resulting in depletion of tumor nutrient and oxygen supply, or (2) a partial inhibition that results in normalization of tumor vasculature, enhances oxygenation and decreases hypoxia-based radiation resistance. Some rationales have been described for combining VEGF and EGFR inhibition. Amongst others, EGFR is also involved in angiogenesis and it has been described that EGFR inhibitor resistance may be associated with VEGF up-regulation and angiogenesis (42, 43).

In this context Bozec et al. demonstrated promising results using the VEGFR inhibitor cediranib (AZD2171) (targeting VEGFR1/2/3) concurrent with the EGFR inhibitor gefitinib and radiotherapy in a VEGF secreting HNSCC xenograft model. Combined treatment plus radiation clearly inhibited tumor growth more effectively than dual or single inhibition or radiotherapy alone. Dual inhibition was associated with decreased vessel density and dual inhibition plus irradiation showed the highest decrease in proliferation as assessed by Ki67 staining (44). The group could confirm the radiosensitizing effects in further studies when treating the same VEGF-secreting HNSCC model as orthotopic xenografts using alternative, but functionally equivalent agents, namely the anti-VEGF monoclonal antibody bevacizumab combined with the EGFR TKI erlotinib or using the combination of the VEGFR TKI sunitinib and the EGFR mAb cetuximab (45, 46). Due to an observed tumor re-growth associated with AKT/mTOR signaling activation, they further investigated the triple-targeting approach of cetuximab, bevacizumab, and the mTOR inhibitor temsirolimus in combination with irradiation. Adding the third inhibitor they indeed achieved the most sustained growth inhibition (47). In previous studies the same group had combined ZD6126, an antivascular tubulin-binding agent, with the EGFR TKI inhibitor gefitinib and irradiation. In contrast to the results described above, and although the combined targeting was moderately more effective than single targeting, the addition of radiation to dual targeting did not result in a further reduction of tumor growth (48).

Radiosensitization could also be induced in a lung cancer model by vandetanib, an inhibitor of VEGFR2 and EGFR but also of RET and other receptors. In human lung adenocarcinoma vandetanib treatment added to radiotherapy resulted in a dose enhancement ratio of 1.32 and markedly inhibited sublethal damage repair as assessed by a split dose recovery assay. *In vivo* the combination with irradiation showed enhanced tumor growth inhibition as compared to single treatment (49). Oehler et al. tested the effect of AEE788, an inhibitor of EGFR, HER2

and VEGFR, plus irradiation in a spontaneously growing murine mammary carcinoma model and in tumor allografts derived from murine mammary carcinoma cells. AEE788 alone as well as in combination with radiation improved tumor oxygenation in both models and the combined treatment resulted in an at least additive tumor response. Using specific inhibitors, the improvement of oxygenation could be assigned to the EGFR/HER2 inhibition (50).

In U87 GBM cell lines with or without ectopic EGFR expression vandetanib as well as cediranib failed to induce radiosensitization in clonogenic assays indicating no effect on DNA repair. In the respective xenograft models only the combination of vandetanib plus irradiation reduced tumor growth more strongly than irradiation alone, and only in the EGFR expressing substrain. In line with reduced tumor growth in this model system, vandetanib but not cediranib suppressed the expression levels of pAkt, survivin, and Ki67 as well as VEGF secretion (51).

The PI3K-AKT-mTOR Pathway

The stimulation of various growth factor receptors leads to the activation of the PI3K-AKT-mTOR signaling pathway, which can cause resistance to apoptosis and radiation. Elevated activity of the PI3K-AKT-mTOR pathway is observed in a broad range of tumor entities and associated with poor outcome, which makes this pathway a promising target for inhibitory strategies (52–55).

mTORC1/mTORC2

Inhibition of the PI3K-AKT-mTOR pathway is usually achieved by mTOR inhibitors, such as rapamycin or everolimus. However, these inhibitors block the mTOR Complex1 (mTORC1), which often results in the up-regulation of the mTOR Complex 2. Therefore, combined inhibition of mTOR Complex 1 and 2 has been studied using dual inhibitors. Sapanisertib is an ATP-competitive mTORC1 and mTORC2 inhibitor. Miyahara et al. demonstrated an enhanced inhibition of proliferation and induction of apoptosis when combining the dual inhibitor and radiation in diffuse intrinsic pontine glioma cells (56). Liu et al. also showed a radiosensitizing effect of sapanisertib in breast cancer cells, which was associated with G2/M cell-cycle arrest and an inhibition of DNA double-strand break (DSB) repair (57).

Hayman et al. compared the radiosensitization through the mTORC1-inhibitor rapamycin and the dual mTORC1/mTORC2 inhibitor PP242 in breast cancer cell lines and only observed a radiosensitizing effect using the dual inhibitor. As a normal tissue cell control, lung fibroblasts were not radiosensitized through PP242 treatment. *In vivo* PP242 alone had no impact on tumor growth but enhanced the radiation-induced growth reduction (58). The same group also tested an alternative mTORC1/mTORC2 inhibitor, AZD2014, which induced radiosensitization in glioblastoma stem-like cells *in vitro* and *in vivo*. A delay in the dispersal of radiation-induced γ H2AX foci suggests that this effect involves the inhibition of DNA repair (59).

PI3K/mTOR targeting

In addition to dual targeting of mTORC1 and mTORC2 the combination of inhibitors targeting different players of the PI3K-AKT-mTOR pathway are under highly intensive investigation. In this context Yu et al. examined the effect of the dual PI3K/mTOR inhibitor dactolisib (NVP-BEZ235) in patient-derived and in radioresistant oral squamous cell carcinoma cells *in vitro* and in an *in vivo* tumor model. They observed radiosensitization *in vitro*, associated with G1 phase arrest by the downregulation of cyclin D1/CDK4 complex as a consequence of the PI3K/mTOR signaling inhibition. Tumor shrinkage was more pronounced upon the combination of dactolisib and radiation as compared to radiation alone (60). Dactolisib was further shown to reduce the activity of the central DNA repair factors DNA-PKcs and ATM and, as a consequence, to efficiently block the repair of IR-induced DSBs. Consequently, an effective radiosensitization could be demonstrated in glioblastoma cells *in vitro* and *in vivo* (61, 62).

Aberrant activation of the PI3K/AKT/mTOR pathway by Ras mutations is an important factor in Ras-driven tumorigenesis (63). Using dactolisib, Konstantinidou et al. could demonstrate a more effective radiosensitization of K-ras mutant NSCLC cells as compared to the single inhibition of PI3K (LY294002) or mTOR (rapamycin). *In vivo* dactolisib alone had little effect on tumor growth but profoundly enhanced the effect of irradiation (64). Substantiating this data, Chen et al. also targeted PI3K and mTOR with dactolisib using K-ras mutant and wild type colorectal cancer cells. Dactolisib had a radiosensitizing effect in both cases. They further demonstrated the same effect in a xenograft tumor model and suggested inefficient DNA repair, possibly due to impaired activation of ATM and DNAPKcs upon dactolisib treatment (65). In glioblastoma cell lines the radiosensitizing effect of dactolisib was shown to be dependent on the scheduling of drug and radiation. A 24 h preincubation period and wash out of the drug right before irradiation and seeding failed to sensitize the cells, while the addition of the drug shortly (1 h) before radiation with subsequent incubation for 24 h before seeding was highly effective. In line with the colony formation data, only the latter schedule showed reduced levels of P-AKT and P-mTOR without and 30 min after irradiation (66). Potiron et al. used dactolisib *in vitro* and *in vivo* in prostate cancer cell lines under normoxic and hypoxic conditions. They found a radiosensitizing effect in all cases and observed a reduction in DSB repair associated with an enhanced G2 cell cycle arrest (67). Comparable results in prostate cancer cell lines were reported in two further studies, supporting the theory of an impaired DNA repair capacity (68, 69). Schötz et al. observed radiosensitization in HNSCC cell lines, regardless of HPV-status. A DNA-repair defect was more apparent in the G1 than G2 phase and reporter gene assays pointed toward inhibition of non-homologous endjoining (NHEJ), but not homologous recombination (HR) (70). Chang et al. also tested an alternative dual PI3K/mTOR inhibitor, PI-103, which caused radiosensitization comparable to dactolisib. They suggested a novel mechanism of radiosensitization based on a reduced expression of NHEJ (Ku70/80), as well as HR (BRCA1/2, Rad51) factors upon PI3K/mTOR inhibition and radiation (69). Along

the same line Jang et al. reported a severely reduced BRCA1 expression upon PI-103 treatment and a radiosensitization that could be further augmented by PARP-inhibition through olaparib. PI-103 failed to induce radiosensitization after a preceding siRNA-mediated knockdown of BRCA1 suggesting that BRCA1/HR is the most relevant target in this regard (71). PI-103 was also shown to radiosensitize colon cancer cells with activated AKT through inhibition of DSB repair (72).

Leiker et al. analyzed a third ATP-competitive dual PI3K-mTOR inhibitor, PF-05212384. Using HNSCC cells they demonstrated delayed γ H2AX foci resolution and a significant radiosensitization *in vivo* and *in vitro*. Since the effect was more pronounced in tumor cells compared to normal fibroblasts the results indicate some degree of tumor specificity (73). A differential response in two HNSCC cell lines toward the PI3K-mTOR inhibitor, PF-04691502 was described by Tonlaar et al. While one strain was sensitized, the other failed to respond, in line with an increased constitutive activity of PI3K, AKT, and mTOR and an inability to inhibit key phosphorylation events upon treatment (74).

Following a concept of PI3K/mTOR inhibition different from the ones described above, Fokas et al. used dactolisib as an alternative to VEGFR-inhibition in order to induce vascular normalization and improved oxygen supply. *In vivo* they observed a reduction in tumor hypoxia and an increase in perfusion. Using different schedules of drug treatment and irradiation that did or did not provide adequate time for vascular remodeling, they observed differences in tumor growth delay and concluded that dactolisib is capable of both, radiosensitization through vasculature normalization and in a direct manner (75). The same group further characterized this direct effect in a panel of different tumor and endothelial cells using dactolisib and another dual PI3K/mTOR inhibitor, NVP-BGT226. They observed PI3K pathway inhibition and enhanced residual γ H2AX foci and G2-arrest after irradiation. Human endothelial and dermal microvascular cells were also sensitized, which suggests possible effects on tumor vasculature but may also indicate sensitization of normal tissue cells, which urges caution, when progressing to clinical trials (76).

AKT/mTOR

Another possibility for highly effective targeting of the PI3K-AKT-mTOR pathway is the combined inhibition of AKT and mTOR. Upon treatment with the mTOR-inhibitor rapamycin, Holler et al. observed an activation of Akt in cell lines that showed no or little radiosensitization. Since this activation was not present in responsive cells, they combined rapamycin with the Akt-inhibitor MK2206 and observed radiosensitization and an enhanced number of residual DSBs (77).

Combined inhibition of the PI3K/AKT/mTOR and Ras/Raf/Mek/MAPK pathways

Since there is crosstalk between the PI3K/AKT/mTOR pathway and the Ras/Raf/MAPK pathway with compensatory potential, dual targeting of these two pathways is also an option. Williams et al. investigated the inhibition of both pathways in K-ras mutated pancreatic cancer cells and xenografts. While sole MEK

inhibition by PD0325901 already resulted in radiosensitization and apoptosis, both effects were further enhanced by a dose of the Akt-inhibitor API-2 that was not effective on its own. Dual inhibition plus radiation also showed the most pronounced growth inhibition in a corresponding xenograft model (78). Toulany et al. demonstrated radiosensitization in K-ras mutated NSCLC cells upon PI-103 treatment but prolonged inhibition resulted in K-ras/Raf/MAPK-dependent Akt activation and loss of radiosensitization. Combining PI3K/mTOR inhibition with the MEK inhibitor PD98059 prevented the reactivation of K-ras/Raf/MAPK-dependent Akt signaling upon long-term PI-103 incubation and resulted in inhibition of DSB repair and radiosensitization (79). Using the MEK inhibitor AZD6244, Kuger et al. investigated whether additional inhibition of the MAPK pathway further enhances the radiosensitization induced by dactolisib treatment. They consistently found a radiosensitizing effect through PI3K/mTOR inhibition in lung and glioblastoma cancer cells that, however, was not increased through additional MEK inhibitor (80). Lastly, Blas et al. combined the PI3K family inhibitor buparlisib with the MEK1/2 inhibitor binimetinib in HNSCC cells. *In vitro*, both inhibitors showed a dose dependent inhibition of proliferation/viability without additional effects upon combination. None of the inhibitors, nor the combination induced radiosensitization, partly even induced radioprotection in UT-SCC-15 cells. *In vivo*, combining both inhibitors did not show any benefit in combination with irradiation and in UT-SCC-15 cells even diminished the growth delay compared to radiotherapy with either agent alone (81).

TABLE 1 | Continued

Targets	Inhibitor(s)	Entity	References	
EGFR/mTOR	Erlotinib ^{**,***} Everolimus ^{**,***}	NSCLC?	(39)	
EGFR/VEGFR	Gefitinib ^{**,***} ZD6126* (sus)	HNSCC	(48)	
	Gefitinib ^{**,***} AZD2171 (Cediranib) ^{**}	HNSCC	(44)	
	Erlotinib ^{**,***} Bevacizumab ^{**,***}	HNSCC	(45)	
	Cetuximab ^{**,***} Sunitinib ^{**,***}	HNSCC	(46)	
	Cetuximab ^{**,***} Bevacizumab ^{**,***} Temozolomide ^{**,***}	HNSCC	(47)	
	Vandetanib ^{**,***} Vandetanib ^{**,***}	NSCLC GBM	(49) (51)	
EGFR/VEGFR/ HER2	AEE788*(disc)	Mammary carcinoma (murine)	(50)	
mTOR1C/mTOR2C	Sapanisertib*	Pontine Glioma Breast Cancer	(56) (57)	
	PP242 ^{exp} AZD2014 (Vistusertib)*	Breast Cancer Glioblastoma	(58) (59)	
PI3K/mTOR [(ATM/DNAPK _{CS})]	Dactolisib*	Oral SCC	(60)	
	Dactolisib*	HNSCC Glioblastoma NSCLC Colorectal cancer Prostate cancer Fibrosarcoma, HNSCC	(70) (61, 62, 66) (64) (65) (67, 68) (75)	
	Dactolisib* NVP-BGT226 *(disc) Dactolisib* PI-103 ^{exp}	HNSCC, bladder cancer, endothelial cells Prostate cancer	(76) (69)	
	PI3K/mTOR	PI-103 ^{exp} PF-05212384 (Gedatolisib)* PF-04691502* (disc)	Colon cancer HNSCC HNSCC	(72) (73) (74)
	PI3K/mTOR/PARP	PI-103 ^{exp} Olaparib ^{**,***}	TNBC	(71)
	mTOR/Akt	Rapamycin ^{**,***} MK2206*	NSCLC, breast cancer	(77)
	MEK/Akt	PD0325901* API-2 (=Triciribine)*	Pancreatic cancer (K-ras mut.)	(78)
	PI3K/mTOR/MEK	PI-103 ^{exp} PD98059 ^{exp} Dactolisib* AZD6244 (Selumetinib) ^{**,***}	K-ras mut. NSCLC Lung cancer, Glioblastoma	(79) (80)
	PI3K/MEK	Buparlisib ^{**} Binimetinib ^{**,***}	HNSCC	(81)

*Tested in clinical trials.

**Tested in clinical trials in combination with radiotherapy.

***Approved (any clinical setting).

exp, experimental; disc, discontinued; sus, suspended.

TABLE 1 | Combined targeting of growth factor receptor signaling.

Targets	Inhibitor(s)	Entity	References
EGFR/HER2	Gefitinib ^{**,***} Trastuzumab ^{**,***}	Vulvar squamous cell carcinoma	(20)
EGFR/HER2	Lapatinib ^{**,***}	Breast cancer Breast cancer (HER2+) K- pancreatic cancer (K-ras wt) NF2 associated peripheral nerve sheath tumor	(21, 22, 24) (25) (23) (26)
		Bladder cancer	(27)
EGFR/HER3	MEHD7945A*	NSCLC, HNSCC	(28, 29)
EGFR/IGF-1R	Panitumumab ^{**,***} Ganitumab*	HNSCC	(31)
	Erlotinib ^{**,***} AG1024 ^{exp} AG1478 ^{exp} AG1024 ^{exp}	Prostate cancer Breast cancer	(32) (33)
EGFR/JAK/STAT-3	Cetuximab ^{**,***} JAK1i ^{exp}	HNSCC	(37)
EGFR/JNK2/JIP-4	Cetuximab ^{**,***} SP600125 ^{exp}	HNSCC/VSCC	(38)

(Continued)

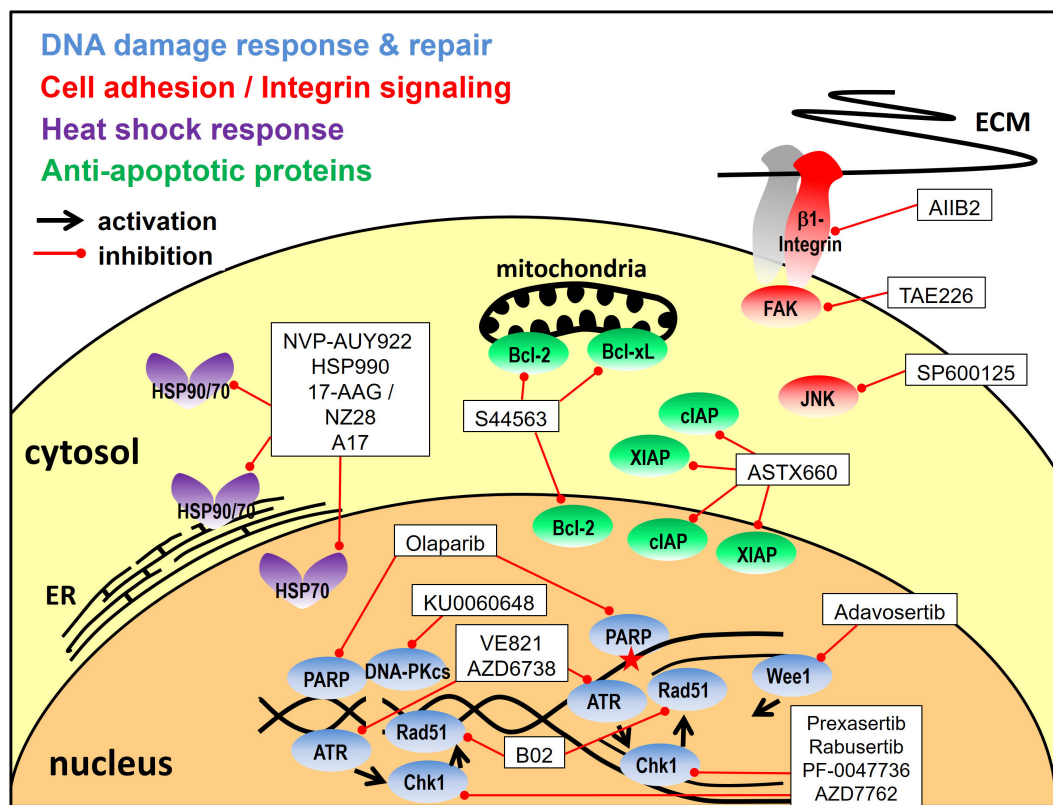


FIGURE 3 | Targeting approaches other than signal transduction pathways. Depicted are the inhibitors utilized for combined molecular targeting of the DNA damage response, integrin signaling, the heat shock response or apoptosis for tumor radiosensitization and the respective target proteins. Reported inhibitor combinations are described in the text and are listed in **Tables 2, 3, 4**. ECM, extracellular matrix; ER, endoplasmic reticulum.

Apart from the inhibition of signal transduction pathways a number of other strategies have been developed for tumor radiosensitization through combined molecular targeting. These include the targeting of the DNA damage response, cell adhesion molecules, the heat shock response or apoptosis, as detailed below and outlined in **Figure 3**.

Targeting the DNA Damage Response

Ionizing radiation causes DNA lesions, such as base damages, single-strand breaks, and double-strand breaks with the latter being largely responsible for cell inactivation (82). Therefore, the most obvious approach for radiosensitization is the direct targeting of the DNA damage response (DDR) and DSB repair. An integral part of the DDR are the damage induced cell cycle checkpoints in the G1, S or G2 phase, which allow additional time for DNA repair before the critical passage through mitosis where mis- or unrepaired DSBs can result in cell death due to failure in chromosome segregation (83). One of the most frequent transforming events in human cancerogenesis is the inactivation of p53. p53 mutations, the overactivation of the MDM2-controlled regulatory pathway or p53 degradation through viral oncoproteins represent the underlying mechanisms (84, 85). As p53 is essential for G1 checkpoint activation its deficiency renders affected tumor cells more dependent on S/G2

cell cycle checkpoint activation (83). Upon DNA damage these checkpoints are activated through checkpoint kinase 1 (Chk1), which is further involved in DNA repair through HR and has an impact on the stabilization of stalled replication forks and other responses to genotoxic stress during the S-phase (86). Upon activation through phosphorylation it inactivates members of the Cdc25 phosphatase family which leads to the inactivation of the cyclin-dependent kinases (CDKs) 1/2 and arrests cells in the G2 phase in response to DNA damage (87). Another kinase necessary for S and G2 checkpoint activation as well as for normal cell cycle progression is Wee1. As the direct counterpart of CDC25 phosphatases it constitutively inactivates CDK1/2 through phosphorylation and is also involved in homologous recombination (88, 89). Targeting the S- and G2-checkpoints through the inhibition of Chk1 and partly of Wee1 has been a frequently used approach for preclinical radio- or chemosensitization and was recently combined with PARP-inhibitors. The rationale is that the inhibition of PARP causes additional DNA damage especially in the S- and G2-phase through the inhibition of single-strand break (SSB) repair and PARP trapping on damaged DNA and through the subsequent collision of single strand lesions with replication forks (90–92). PARP-inhibition further impairs the alternative end-joining pathway which is also preferentially active in S- and G2 (93).

These mechanisms additionally enhance the dependence on the S- and G2-checkpoints and the well described synthetic lethality of PARP-inhibition and HR deficiency (94) may further increase radiosensitization.

Chk1/Wee1

Focusing on cell-cycle checkpoint inhibition, Busch et al. tested the combined targeting of Chk1 (LY2603618) and Wee1 (adavosertib; AZD1775) in HPV-positive HNSCC cells because they had observed an activation of Chk1 upon Wee1 inhibition that may in part counteract the effects of sole Wee1 targeting. Analyzing proliferation, inhibition of G2 arrest and radiosensitization, they found dual targeting to be effective at profoundly reduced concentrations as compared to single agent usage. Additionally, they observed only minimal radiosensitization in p53 proficient normal human fibroblasts, thus demonstrating tumor specificity (95).

PARP1/Chk1

Vance et al. combined the inhibition of PARP1 through olaparib and Chk1 through AZD7762 in p53 mutant pancreatic cancer cells and observed an additive radiosensitization. The authors observed G2 checkpoint abrogation, inhibition of HR and a persistent γ H2AX signal after combined inhibition of the two targets. There was no significant radiosensitization in G1-checkpoint-proficient intestinal epithelial cells, backing up the hypothesis that tumor cells harboring aberrations in p53 or other DNA damage response pathways are more selectively sensitized (96). In line with these data, Güster et al. demonstrated radiosensitization of p53 deficient HPV-positive HNSCC cells through olaparib and the Chk1-inhibitor PF-0047736, with the extent of sensitization being highest upon combined inhibition (97).

PARP/Wee1

Karnak et al. investigated the radiosensitizing effect of the combined inhibition of PARP1 and Wee1 through olaparib and adavosertib in pancreatic cancer cells. This dual-targeted approach is highly similar to combined PARP/Chk1-inhibition and was also associated with G2 checkpoint abrogation, inhibition of HR and persistent DNA damage. *In vitro* the combination of both inhibitors caused enhanced radiosensitization as compared to single inhibition. *In vivo*, there was no radiosensitization with olaparib alone and a moderate effect of adavosertib. Combined targeting, however, demonstrated highly significant radiosensitization (98). The same group further assessed this dual-targeting approach in hepatocellular carcinoma cells and K-ras mutant NSCLC cells, also showing an increased radiosensitization *in vitro* and *in vivo* compared to either agent alone. The authors suggested that trapping of PARP to chromatin by olaparib as well as replication stress induced through this inhibitor combination contribute to radiosensitization (99, 100). Molkentine et al. compared PARP-inhibition through niraparib plus either Wee1-inhibition through adavosertib or Chk1-inhibition through MK-8776 in an HPV-positive and an HPV-negative cell line. While both ways of S/G2-checkpoint-inhibition enhanced the radiosensitization

through sole PARP-inhibition, the addition of Chk1-inhibition was more effective in the HPV-positive and of Wee1-inhibition in the HPV-negative strain. Whether these differences are generally valid for the two subentities remains to be shown in future studies (101).

PARP/ATR

Carruthers et al. had reported that glioblastoma stem-like cells are characterized by intrinsic replication stress, which activates the DDR and leads to radiation resistance. Ataxia telangiectasia and Rad3-related protein (ATR) is a key DDR kinase acting directly upstream of Chk1. Through Chk1 activation but also partly independent from Chk1, ATR is critically involved in replication processes, such as the stabilization of stalled replication forks, and in DSB repair pathways (102, 103). Targeting the replication stress response by a combination of olaparib and the ATR inhibitor VE821 resulted in cytotoxicity and synergistic radiosensitization, completely abolishing radioresistance (104). These data confirm results from a previous report by the same group, where the same combination resulted in greater radiosensitization than ATM inhibition in primary glioblastoma cell cultures. Radiosensitization was higher when the cells were cultured under conditions enriching the fraction of stem-like cells as compared to conditions favoring their depletion and a more differentiated state (105).

PARP/Rad51

Olaparib was further combined with the Rad51 inhibitor B02 and X- as well as proton-irradiation with the intention to induce HR deficiency that would synergize with PARP inhibition. Lung and pancreatic cancer cell lines were radiosensitized by the inhibitors, with the strongest effect for dual inhibition, similarly for both types of irradiation. Radiosensitization was found to be dependent on the proliferation rate, as serum deprivation reduced the effectiveness of dual targeting and in slowly proliferating PANC1 cells the combination was even less effective than sole PARP-inhibition (106).

Chk1/2/EGFR

The addition of the Chk1/2 inhibitor prexasertib to cetuximab and irradiation was investigated by Zeng et al. in HPV-positive and HPV-negative HNSCC cell lines. Prexasertib caused an accumulation of cells in the S-phase, the triple combination partly resulted in decreased proliferation and increased apoptosis as compared to single or double treatment (107).

ATR/DNA-PK

DNA-dependent protein kinase, catalytic subunit (DNA-PKcs) is well known as an essential component of the classical NHEJ pathway but is further associated with genomic stability, hypoxia, inflammatory responses, metabolism and regulation of transcription (108, 109). Hafsi et al. used combined ATR and DNA-PKcs inhibition (AZD6738, KU0060648) to radiosensitize HNSCC cells and observed an at least additive effect. A key element in this approach is that ATR inhibition interferes with cell cycle arrest and HR, whereas DNA-PKcs inhibition inhibits NHEJ. This combination therefore leaves few options for the cells

to repair the radiation-induced damage in any cell cycle phase and curbs the development of resistance mechanisms. It may, however, come at the cost of tumor specificity (110).

Targeting Cell Adhesion Molecules

Cell matrix interaction by integrins was shown to be a modulator of tumor progression, invasion, metastasis and response to therapy. β 1-integrin, a member of the integrin family of cell adhesion molecules is significantly involved in tumor survival and proliferation and is associated with radio- or chemotherapy resistance (111). β 1-integrin overexpression was shown in many tumor entities and its molecular targeting was found to be an effective means of radiosensitization. Integrins recruit signaling molecules to their cytoplasmic domain, mainly focal adhesion kinase (FAK) but also components of the EGFR signaling pathway, such as Erk and Akt (112). FAK is involved in proliferation, cell motility and radiation response and was found to be overexpressed or hyperphosphorylated in e.g., liver, head, and neck or breast cancer cells.

β 1 Integrin or FAK/EGFR

Eke et al. investigated the effect of concurrent β 1 integrin and EGFR targeting using the monoclonal inhibitory antibodies AIIB2 and cetuximab, respectively in head and neck cancer cells. They observed enhanced cytotoxicity and radiosensitization upon combined inhibition in 8 out of 10 cell lines and, in line

with that, enhanced survival in a xenograft model of a responder cell line (113). FAK was shown to mediate the effects of β 1 integrin targeting in line with previous reports of the same group that had shown dual inhibition of EGFR (cetuximab, siRNA) and FAK (TAE226, siRNA) to achieve a stronger radiosensitizing effect in HNSCCs than either inhibitor alone (114). Zscheppang et al. further investigated single and dual β 1-integrin/EGFR targeting using AIIB2 & cetuximab in sphere-forming HNSCC cells based on the concept that tumor initiating cells are enriched in spheres. Sphere-forming cells were found to be resistant to this targeting approach and future work is warranted to understand the mechanisms and relevance of this finding (115). In another report, the same dual β 1-integrin/EGFR inhibition approach, as well as KRAS or BRAF depletion and 5-FU-treatment failed to modulate the radiosensitivity of colorectal carcinoma cells (116).

Recently, a screen for predictive biomarkers for the dual β 1-integrin/EGFR targeting approach showed different mutational profiles of responding and non-responding cells and suggested some proteins as potential resistance factors. Using an RNAi screen and pharmacological inhibition (ML334, everolimus) Kelch like ECH associated protein 1 (KEAP1) and mTOR were identified as druggable targets for radiosensitization in combination with β 1-integrin/EGFR targeting (117).

β 1-Integrin/c-Abl

C-Abl is a tyrosine kinase found to be hyperphosphorylated upon β 1-integrin inhibition. Therefore, dual β 1-integrin (AIIB2) and c-Abl (imatinib) targeting was tested in a panel of tumor cell lines from various entities, where a cell line dependent cytotoxicity and enhancement or induction of radiosensitivity was observed as compared to single treatment in a subgroup of the panel. Radiosensitization was accompanied by altered expression of DSB repair proteins KU70 and NBS1 and was associated with reduced DSB repair (118).

TABLE 2 | Dual targeting of DNA damage response factors.

Targets	Inhibitor(s)	Entity	References
Chk1/Wee1	LY2603618 (Rabusertib) ^{*(disc)} Adavosertib ^{**}	HNSCC (HPV+)	(95)
PARP1/ Chk1	Olaparib ^{**,***} AZD7762 ^{*(disc)}	Pancreatic cancer (p53 mut)	(96)
	Olaparib ^{**,***} PF-0047736 ^{*(disc)}	HNSCC (HPV+)	(97)
		HNSCC	(101)
PARP1/Wee1	Olaparib ^{**,***} Adavosertib ^{**}	Pancreatic cancer	(98)
		Hepatocellular carcinoma	(99)
		NSCLC (K-ras mut)	(100)
		HNSCC	(101)
PARP1/ATR	Olaparib ^{**,***} VE821 ^{exp}	GBM	(104, 105)
PARP1/RAD51	Olaparib ^{*,***} B02 ^{exp}	NSCLC, Pancreatic cancer	(106)
Chk1/2/EGFR	Prexasertib ^{**} Cetuximab ^{**,***}	HNSCC	(107)
ATR/DNA-PK	AZD6738 (Ceralasertib) [*] KU0060648 ^{exp}	HNSCC, Colon cancer	(110)

^{*}Tested in clinical trials.

^{**}Tested in clinical trials in combination with radiotherapy.

^{***}Approved (any clinical setting).

exp, experimental; disc, discontinued.

TABLE 3 | Combined targeting approaches involving cell adhesion molecules.

Targets	Inhibitor(s)	Entity	References
β 1-integrin/ EGFR	AIIB2 ^{exp}	HNSCC	(113)
	Cetuximab ^{**,***}	HNSCC	(115)
		Colorectal Cancer	(116)
β 1-integrin/ EGFR/KEAP1/ mTOR	AIIB2 ^{exp}	HNSCC	(117)
	Cetuximab ^{**,***} Everolimus ^{**,***} ML334 ^{exp}		
FAK/EGFR	TAE226 ^{exp} Cetuximab ^{**,***}	HNSCC	(114)
β 1-integrin/ c-Abl	AIIB2 ^{exp} Imatinib ^{***}	various	(118)
β 1-integrin/ JNK	AIIB2 ^{exp} SP600125 ^{exp}	Glioblastoma	(119)

^{**}Tested in clinical trials in combination with radiotherapy.

^{***}Approved (any clinical setting).

exp, experimental.

β 1-Integrin/JNK

Vehlow et al. identified the c-Jun N-terminal kinase (JNK), a known stress mediator, to mediate bypass signaling after β 1-integrin-inhibition in established glioblastoma cell lines, as well as stem-like and patient-derived glioblastoma cells. Dual β 1-integrin/JNK inhibition through AIIB2 and the JNK inhibitor SP600125 *in vitro* and *in vivo* resulted in a superior effect when combined with radiation as compared to single inhibition, i.e., increasing the median survival of orthotopic, radiochemotherapy-treated GBM mice. *In vitro* the authors observed defects in DNA repair associated with chromatin changes, enhanced ATM phosphorylation, and prolonged G2/M cell cycle arrest as the underlying mechanism of radiosensitization (119).

Targeting the Heat-Shock Response

Heat shock proteins (HSPs) are a group of proteins with enhanced expression in response to various kinds of stresses, such as hyperthermia, infections, heavy metals, or oxidative stress (120). As molecular chaperones they assist their substrate proteins, termed clients, in acquiring or recovering their functional three dimensional fold, a process especially important under stressed conditions. Furthermore they assist the binding of ligands to their targets and the assembly of multiprotein complexes and they are potent inhibitors of apoptosis (121, 122). HSP70 and HSP90 proteins represent two important, druggable HSP families with actually hundreds of client proteins making their molecular targeting a biologically complex approach with numerous possible subsequent effects. The inhibition of both HSP70 and HSP90 are being tested for cancer therapy because of their especially high expression levels in tumors. Enhanced expression is believed to be necessary because in tumor cells proteostasis is permanently challenged by tumor cell metabolism, oxidative stress, dysregulated protein expression and the expression of mutant (onco) proteins, which may be less stable and require more assistance from the chaperone machinery (123). A prominent example for the latter is the stabilization of mutant, gain of function p53 variants through HSP90 (124).

HSP90/HSP70

It was shown that the inhibition of Hsp90 compromises DNA repair after irradiation and enhances tumor cell radiosensitivity (125, 126). However, HSP90 inhibition also leads to the activation of the transcription factor Heat Shock Factor 1 (HSF 1). HSF-1 is inactivated when bound by HSPs and becomes active upon release, e.g., upon HSP-inhibition or under stressed conditions, in order to adjust the HSP-expression level to the chaperone demand of the cell (120). Therefore, targeting HSP90 can enhance the expression of Hsp70, which may partly antagonize the effects of HSP90-inhibition. This led to the dual targeting approach of Schilling et al. in which HSP70 inhibition through the peptide aptamer A17 failed to significantly radiosensitize lung and breast cancer cells on its own but augmented the radiosensitizing effect of the Hsp90 inhibitor NVP-AUY922. The authors suggested that increased levels of DNA double-strand breaks and enhanced G2/M arrest are involved in cell death after combined treatment and radiation (127). In a previous work the

same group had already shown similar results in which addition of NVP-AUY922 allowed for a reduction in the concentration of the HSP70 inhibitor NZ28 to 1/10 to 1/20 to still achieve the same radiosensitization (128).

HSP90/PI3K/mTOR

Following the same concept as dual HSP70/90-inhibition the PI3K/mTOR inhibitor PI-103, which had previously been shown to suppress the up-regulation of Hsp70 (129), was combined with Hsp90-inhibition through NVP-AUY922. Adding both inhibitors 3h prior to irradiation followed by 24h of culture moderately enhanced the radiosensitizing effect. The authors supposed a down regulation of PI3K and ERK pathways, increased DNA damage, and a pronounced G2/M arrest as possible causative factors. Interestingly, using another treatment schedule, adding the inhibitor 24h before irradiation slightly reduces the radiosensitizing effect of the HSP90 inhibitor. They considered a reactivation of the PI3K/MAPK pro-survival pathway and an increased G1 arrest at the moment of irradiation and better DNA repair to cause these controversial observations. These findings underline the importance of IR-drug scheduling (130).

In human glioma cells Wachsberger et al. combined the PI3K inhibitor Buparlisib (BKM120) with the HSP90 inhibitor HSP990, which resulted in downregulation of the AKT pathway and induction of apoptosis. *In vitro* from a panel of four cell lines only U373MG showed a profound radiosensitization after dual targeting as compared to single inhibition. Still, *in vivo*, U87MG showed a more pronounced tumor growth delay compared to single inhibition with and without the combination with irradiation (131).

HSP90/PARP1

Also targeting human glioma cells Dungey et al. combined the inhibition of PARP through olaparib with the Hsp90 inhibitor 17-AAG. The rationale behind is that Hsp90-inhibition

TABLE 4 | Combined targeting approaches involving the heat shock response.

Targets	Inhibitor(s)	Entity	References
HSP90/HSP70	NVP-AUY922 (Luminespib) ^{exp} NZ28 ^{exp}	Lung and breast cancer	(128)
	NVP-AUY922 ^{exp} A17 (peptide aptamer) ^{exp}	Lung and breast cancer	(127)
HSP90/PI3K/mTOR	NVP-AUY922 ^{exp} PI-103 ^{exp}	Glioblastoma, colon cancer	(130)
HSP90/PI3K	HSP990 ^(disc) Buparlisib ^{**}	Glioma	(131)
HSP90/PARP	17-AAG (Tanespimycin) ^(disc) Olaparib ^{***,***}	Glioma	(132)

^{*}Tested in clinical trials.

^{**}Tested in clinical trials in combination with radiotherapy.

^{***}Approved (any clinical setting).

exp, experimental; *disc*, discontinued.

decreases HR, which is needed to repair replication-associated DSB generated through PARP inhibition. They observed a downregulation of Rad51 and BRCA2 protein levels and inhibition of HR upon HSP90 inhibition. Combined treatment resulted in additive radiosensitization in proliferating cells. Since the authors did not observe radiosensitization through HSP90 inhibition in non-tumor control cells and had previously described olaparib-mediated radiosensitization to be replication-dependent, they expect an enhancement of the therapeutic ratio by taking advantage of the non-dividing state of normal brain tissue (132).

Other Approaches

MEK/Cyclin Dependent Kinases (CDKs)

Tao et al. observed that in Kras-mutant NSCLC cells, the inhibition of MEK through trametinib resulted in p16 expression and reduced phosphorylation and therefore activation of the tumor suppressor RB in the cell line most sensitive toward both sole MEK-inhibition and MEK-inhibition induced radiosensitization. Likewise, activation of RB through CDK4/6-inhibition through palbociclib sensitized the more resistant cells to MEK-inhibition and resulted in enhanced radiation sensitivity as compared to single treatment. Dual targeting plus irradiation was also most effective in a xenograft model (133).

Targeting Histone Deacetylases/HER Family Receptors

Histone deacetylase inhibitors are a heterogeneous group of epigenetic therapeutics, which a.o. interfere with DNA damage signaling and repair (134). Moertl et al. have compared the radiosensitization of pancreatic cancer cells through the HDAC inhibitor SAHA and the multi target inhibitor CUDC-101, which, besides HDAC, also targets EGFR and HER2 (135). They observed reduced proliferation and clonogenic survival and increased apoptosis with reduced expression of the antiapoptotic proteins XIAP and survivin with both inhibitors. While the multi target inhibitor was identified as the more potent radiosensitizer, no clues can presently be drawn regarding a synergistic mechanism of HDAC and EGFR/HER2 targeting since no combined treatment of SAHA and HER family receptor inhibition was performed.

Targeting Anti-apoptotic Proteins

We further identified two studies, which followed a strategy of inhibiting two anti-apoptotic proteins. In the first study, Bcl-2 and Bcl-XL, two members of the anti-apoptotic fraction of the Bcl-2 family of mitochondrial membrane proteins, were inhibited using the dual inhibitor S44563. Upon targeting plus irradiation the authors observed an enhanced sub-G1 fraction and caspase 3 cleavage as compared to single treatment. In clonogenic assays they further observed radiosensitization and a slight growth delay in xenograft models upon inhibition plus radiation. Interestingly, treatment was most effective when the inhibitor was added after completion of fractionated radiation, highlighting the importance of the optimal sequence of modalities (136). Another approach utilized a novel antagonist

of the E3 ubiquitin ligases cIAP and XIAP (cellular inhibitor of apoptosis protein, X-linked inhibitor of apoptosis protein), ASTX660. *In vitro*, the inhibitor sensitized subsets of HPV-negative and HPV-positive HNSCC cell lines to TNF family death ligands TNF α and TRAIL, which involved a reactivation of p53 in the HPV-positive strains. In HPV-positive and -negative human HNSCC xenografts the authors observed significantly delayed growth when the dual inhibitor was combined with radiation, which was attenuated by anti-TNF α pretreatment blockade (137).

TABLE 5 | Various combined targeting approaches.

Targets	Inhibitor(s)	Entity	References
MEK/CDK4/6	Trametinib ^{**,***} Palbociclib ^{**,***}	NSCLC (K-ras mut.)	(133)
HDAC/EGFR/ HER2	CUDC-101*	Pancreatic Cancer	(135)
Bcl-2/Bcl-XL	S44563 ^{exp}	SCLC	(136)
cIAP (BIRC2)/XIAP	ASTX660 ^{**}	HNSCC	(137)

*Tested in clinical trials.

**Tested in clinical trials.

***Approved (any clinical setting).

CLINICAL TRIALS

Despite a plethora of positive preclinical data, molecular targeting for tumor radiosensitization is not yet a valid treatment option in the clinic, but a considerable number of clinical trials is testing targeting approaches in combination with (chemo)radiation. However, when searching for trials with combined molecular targeting for radiosensitization in the narrow sense, we only found two running studies combining two inhibitors with sole radiotherapy, both in HNSCC. One trial is testing the combination of cetuximab and the CDK4/6 inhibitor palbociclib, a combination, for which we did not find a preceding preclinical evaluation but only a similar approach combining MEK-inhibition plus palbociclib (133) (NCT03024489). The other trial compares the dual targeting of EGFR and Chk1 through cetuximab and prexasertib vs. the combination of cisplatin and prexasertib (NCT02555644). Since this design does not include single inhibition or standard treatment but uses the non-approved inhibitor prexasertib in both arms, it may become difficult to finally estimate to what extent this dual targeting approach may increase radiation sensitivity.

In the following we present a selection of relevant publications reporting results from clinical trials using combined molecular targeting and (mostly chemo-)radiation. A common approach is the combination of chemoradiation, inhibition of signal transduction pathways and VEGFR-inhibition. However, while these combined inhibitor approaches clearly cover anticipated effectiveness through dual targeting, the purpose of radiosensitization is less in focus than the idea of achieving

additive effects through repression of angiogenesis. Especially the combination of chemoradiation, EGFR-inhibition and the anti-VEGF antibody bevacizumab has been tested in different entities but efficacy so far appears limited: In HNSCC, the addition of bevacizumab to radiation, cetuximab and pemetrexed was reported to increase toxicity without an apparent improvement in efficacy (138). Similarly, the combination of (chemo)radiation, bevacizumab and erlotinib did not result in a survival benefit but demonstrated targeted-agent specific toxicity in esophageal cancer (139). Along the same line, adding erlotinib to chemoradiation and bevacizumab did not show efficacy but induced esophageal toxicity in NSCLC (140) and the addition of the EGFR/VEGFR inhibitor vandetanib did not prolong survival in a phase II study of glioblastoma (141). In contrast to these clearly negative results, encouraging responses have been reported for the addition of erlotinib to neoadjuvant chemoradiation plus bevacizumab in phase I trials of rectal cancer, which warrant further investigation in larger studies (142, 143). Clinical results have also been reported for the inhibition of EGFR and HER2 in HNSCC through lapatinib. Addition to primary chemoradiation plus lapatinib maintenance resulted in increased 6-month complete response rates and progression-free survival as compared to placebo in a phase II study (144). However, in a similar design in the setting of adjuvant chemoradiation after surgery, lapatinib did not result in any efficacy benefits but additional toxicity in a large phase III trial of 688 patients (145).

At present, molecular targeting is often added to current chemoradiation regimes to increase efficacy and trials are mostly in early clinical development. It is therefore not surprising that combined targeting approaches are still quite rare and often based on dual specific inhibitors, which may be easier to implement than inhibitor combinations for which toxicity data may still be lacking, even without radiotherapy.

DISCUSSION

Molecular targeting approaches for tumor radiosensitization have been investigated for two decades (146–149), but their implementation into the clinic has proven extremely difficult. As outlined above, the only molecular targeting agent that is approved in the curative setting in combination with radiotherapy is the anti-EGFR-antibody cetuximab in HNSCC, and considerable doubts exist regarding its efficacy (150). The various approaches described in this review aimed to achieve a meaningful radiosensitization through combined inhibition of two or more targets, mostly with the aim of a more complete pathway inhibition or the suppression of compensatory mechanisms, partly with similarities to the concept of synthetic lethality (**Supplementary Figure 1**). The diversity of the approaches reflects the heterogeneity of radiosensitization strategies although some additional emerging concepts, such as interference with NAD^+ -, glucose- or mitochondrial metabolism (151, 152) or the eradication of cancer stem cells (153, 154) were not identified in our search for combined targeting approaches. While molecular targeting is

also increasingly being considered as a strategy to enhance the efficacy of particle irradiation (155), we only identified one such publication, which reported the effect of PARP and Rad51 inhibition when added to proton (and photon) irradiation (106).

With 49 identified publications, the combined targeting of classical kinase dependent signal transduction pathways, such as EGFR, MAPK, or PI3K/AKT/mTOR signaling is the most exhaustively studied approach. The underlying rationale is that tumor cells often rely on the hyperactivation of these kinases to drive key mechanisms such as proliferation, survival and, to some extent, DNA repair (“oncogene addiction”). This should make them more sensitive to kinase inhibitors than normal tissue, providing some tumor specificity. What remains a major challenge is the choice of pathway inhibition for individual tumors, which requires reliable biomarkers. Unfortunately, the commonly analyzed kinase expression level is a poor surrogate for actual kinase activity, as we have recently demonstrated for EGFR activation in HNSCC (156). Keeping this in mind, it will be crucial to establish robust markers of aberrantly high activity, e.g., the detection of activating mutations, protein phosphorylation levels, or functional measurements. Given the identification of an overactive pathway, dual inhibition may be an appropriate way to achieve highly effective inhibition or to avoid bypass signaling through compensatory pathways or mutations of downstream pathway members. To what extent and in which setting a more effective inhibition of signal transduction pathways will subsequently also translate into a clinically meaningful radiosensitization and finally enhanced patient survival remains to be shown.

A major advantage for the targeting of DDR components is their direct involvement in radiation-induced DNA repair. On one hand, this makes it likely that a majority of tumors will be affected. On the other hand, specificity can be a major issue, as normal cells utilize the same pathways for DNA damage recognition, processing and repair. Nine of the 13 studies identified in this field combined PARP- and S/G2 phase checkpoint-inhibition. This approach is partly based on the model of synthetic lethality (see **Supplementary Figure 1**), which has been described for PARP inhibition and HR deficiency (157), as the inhibition of Chk1, Wee1, and ATR was reported to compromise HR (89, 158–160). The same concept applies to the direct targeting of the central HR factor Rad51 combined with PARP inhibition (106) and in part HSP90 inhibition plus PARP inhibition (132). As HR is only active in the S/G2 phase, some degree of tumor specificity can be expected because normal tissue cells mostly do not proliferate and, in contrast to the majority of tumor cells, are p53 proficient and therefore able to arrest in the G1 phase after irradiation. Additional S/G2 phase-derived DNA damage through PARP inhibition is likely to further increase the dependence on S/G2 arrest, which may further be fostered by oncogenic replication stress.

Similar characteristics, i.e., high pathway activity and tumor cells' reliance also motivate the targeting of adhesion molecules [e.g., high expression of focal adhesion signaling receptors (112)] or the heat shock response [e.g., proteostatic control of instable mutants (123)] in order to achieve

tumor specificity. Again, the additional inhibition of compensatory factors provides a main rationale for dual targeting approaches.

The tailored use of molecular targeting agents based on individual tumor characteristics is referred to as precision oncology. Apart from enhancing efficacy and thereby cure rates, molecular targeting is also expected to reduce toxicity, in case it can replace chemotherapy. It has to be noted, however, that the use of targeting agents can result in considerable side effects, which can of course be more severe and difficult to predict when agents are combined and added to (chemo-) radiotherapy. For example, EGFR inhibition frequently causes skin rash and diarrhea (161), which can be especially severe in the radiation field, when the effects add up with (chemo-) radiation induced erythema/mucositis (162). In a phase 3 study for HNSCC the addition of cetuximab to cisplatin-based chemoradiation resulted in considerably more grade 3/4 mucositis and rash and hence higher rates of interruptions in radiation therapy without achieving any clinical benefit (8). As further examples, combining bevacizumab with concomitant radiotherapy can lead to decreased wound healing (163, 164) and, in patients with lung cancer, to fistula formation (165), and the Chk1 inhibitors LY2603618 and ADZ7762 increased the risk of severe thromboembolic events (166) and cardiac side effects (167), respectively, in part when combined with chemotherapy. At present, preclinical data on tumor radiosensitization hardly ever contain thorough *in vivo* analyses of side effects other than weight loss and inspection of the skin/mucosa in the radiation field. Future approaches should therefore not only focus on the identification of the most efficacious radiosensitization but also more deeply on the safety of a possible clinical translation. Detailed *in vivo* studies on systemic as well as in-field toxicity may help design the most promising clinical trials and achieve better clinical outcomes.

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In conclusion, dual targeting for tumor radiosensitization has shown promising results in pre-clinical studies. The way to proceed toward a substantial future clinical benefit requires convincing *in vitro* mechanistic studies that should ideally include predictive biomarkers, with the results substantiated in a relevant number of adequate model systems, such as cell lines and (patient derived) xenografts, but possibly also tumor stem cell cultures as well as *ex vivo* cultured tumor tissues. The most promising combined targeting approaches should be thoroughly inspected for treatment efficacy and safety based on normal tissue toxicity *in vivo*. Such a concept should lead to the identification of effective targeting strategies for subsets of tumors, based on reliable predictive biomarkers to provide the best possible preclinical rationale to allow clinicians to implement the most appropriate combined targeting strategies in well-designed clinical trials.

AUTHOR CONTRIBUTIONS

KH and TR: idea, literature search, and preparation of tables and figures. All authors were involved in the preparation of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fonc.2020.01260/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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