

Review

## Novel Radiosensitizing Anticancer Therapeutics

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**Abstract.** *In cancer treatment, radiation therapy is second only to surgery in terms of its curative potential. However, radiation-induced tumor cell death is limited by a number of factors, including the adverse response of the tumor microenvironment to radiation treatment and tumor-acquired mechanisms of evasive resistance. Recent attempts to enhance the therapeutic efficiency of ionizing radiation have produced promising results. In this review article, we discuss the development of novel therapeutic strategies for tumor sensitization to radiation therapy. These innovative approaches incorporate the involvement of the immune response and the role of cancer stem cells, as well as direct targeting of signal transduction pathways. Taken together, these concerted efforts demonstrate that the augmentation of radiotherapeutic efficacy results in significantly improved control not only of local disease, but also of metastatic spread and improved overall patient survival.*

Radiation therapy has long been accepted as an effective form of conventional cancer treatment. Delivered alone or in combination with chemotherapeutic agents, ionizing radiation is routinely used to treat tumors of the brain, breast, lung, colon, and reproductive system. In addition, patients suffering from head and neck cancer, melanoma, and pancreatic cancer are also frequent recipients of radiotherapy (1, 2). Despite its initial efficacy, however, local in-field relapse continues to be problematic for a variety of malignant tumors (1).

In patients diagnosed with pancreatic cancer, one of the most lethal tumor types, local persistence and disease

progression still present an obstacle even after high-dose radiation therapy (2). Results from an autopsy study of 18 patients with early surgically resected pancreatic cancer showed that 89% displayed signs of local disease at the time of death (2, 3). While four out of the 18 patients died of metastatic disease in the absence of local progression, it is important to note that 28% of patients with unresectable locally advanced pancreatic cancer died without any evidence of metastatic spread (2, 3). For other radiation-treated malignancies, such as breast and prostate cancer, local relapse is not always so apparent (1, 4, 5). Indeed, tumors at these sites often exhibit complete response immediately after treatment, only to undergo post-radiotherapy recurrence more than ten years later (1, 4, 5). Therefore, tumor radioresistance resulting in poor control of local and metastatic disease, and delayed local relapse in tumors that once received curative doses of ionizing radiation, highlight the need for improved methods of tumor radiosensitization (Table I).

### Targeting Signal Transduction

One key area that is fundamental to enhanced efficacy of radiotherapy involves the radiation-induced signaling cascades that affect the cellular response to radiation. When ionizing radiation is administered to living tissues, highly reactive atoms, molecules, or ions with unpaired electrons are generated. These exceedingly reactive substances are termed free radicals, and they can interact with DNA, proteins, and cell membranes to stimulate complex signal transduction (6). Evidence of the interaction between ionizing radiation and cell membranes can be observed with the activation of the acid sphingomyelinase-dependent signal transduction pathway in a variety of cell types. In irradiated cells, acid sphingomyelinase facilitates the enzymatic hydrolysis of the phosphodiester bond of sphingomyelin to yield the pro-apoptotic messenger ceramide (6-9). This direct relationship between irradiation and cell death through apoptosis illustrates the importance of deducing the circuitous signaling networks that are affected by radiation therapy.

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Table I. Current radiosensitizing agents and their respective targets.

Therapeutic agent	Target	Treated cells or tumors	Stage of clinical development	References
Arachidonyltrifluoromethyl ketone (AACOCF <sub>3</sub> )	Cytosolic phospholipase A2 (cPLA <sub>2</sub> )	Vascular endothelial cells, non-small cell lung cancer, and ovarian carcinoma	Pre-clinical	(12-14)
Methyl arachidonyl fluorophosphonate (MAFP)	Cytosolic phospholipase A2 (cPLA <sub>2</sub> )	Vascular endothelial cells	Pre-clinical	(12)
Bromophosphonate analogue of lysophosphatidic acid (BrP-LPA)	Autotaxin (ATX) and lysophosphatidic acid (LPA) receptors	Vascular endothelial cells, breast cancer, non-small cell lung cancer, and glioblastoma	Pre-clinical	(27, 32)
A12, monoclonal antibody to IGF-1R	Insulin-like growth factor 1 receptor (IGF-1R)	Non-small cell lung cancer	Pre-clinical for radiation; phase I/II clinical trials for non-irradiation	(33, 41)
CP-751,871, monoclonal antibody to IGF-1R	Insulin-like growth factor 1 receptor (IGF-1R)	Non-small cell lung cancer	Pre-clinical for radiation; phase I/II clinical trials for non-irradiation	(42)
AG1024, antibody to IGF-1R	Insulin-like growth factor 1 receptor (IGF-1R)	Glioblastoma, breast cancer	Pre-clinical	(43)
Nutlins	p53 and MDM2 interaction	Laryngeal carcinoma, prostate cancer, and non-small cell lung cancer	Pre-clinical	(6, 62-70)
Diethylaminobenzaldehyde Veliparib	Aldehyde dehydrogenase 1 Poly(ADP-ribose) polymerase (PARP)	Breast cancer stem cells Melanoma, pancreatic cancer, glioma, non-small cell lung cancer, breast cancer	Pre-clinical Clinical trials (phase I/II)	(1, 92) (99)

*Cytosolic phospholipase A2.* Independently of the cellular consequences experienced by irradiated tumor cells, it is imperative to analyze the response of the tumor microenvironment to ionizing radiation. Upon detailed investigation, multiple reports have concluded that the effectiveness of radiotherapy is often limited by the response of tumor vascular endothelium (10-14). Studies by our laboratory demonstrated that the administration of ionizing radiation (3 Gy) to vascular endothelial cells resulted in the activation of cytosolic phospholipase A2 (cPLA<sub>2</sub>) (12-14). cPLA<sub>2</sub> is an 85-kDa Ca<sup>2+</sup>-sensitive protein that belongs to a PLA<sub>2</sub> superfamily. This family of enzymes is responsible for the hydrolysis of the sn-2 acyl bond of glycerophospholipids on the cell membrane (15). Radiation-induced activation of cPLA<sub>2</sub> in vascular endothelial cells resulted in the generation of lysophosphatidylcholine (LPC), a lipid-derived second messenger that triggered Akt and extracellular signal-regulated kinases 1 and 2 (ERK1/2) phosphorylation (12-14). Inhibition of cPLA<sub>2</sub> using pharmacological agents (Table I) or small hairpin RNA (shRNA) specific for cPLA<sub>2</sub> enhanced radiation-induced cell death, characterized by mitotic catastrophe, followed by a delayed programmed cell death (14).

Furthermore, in radioresistant mouse lung tumor models, cPLA<sub>2</sub> inhibition combined with radiation treatment significantly reduced overall tumor blood flow and vascularity and suppressed tumor growth (12). Accompanying data from lung carcinoma and glioblastoma

models using cPLA<sub>2</sub>α-deficient mice revealed that tumors failed to form a functional tumor vascular network, resulting in a dramatic decrease of tumor volume (16). Thus, the presence of cPLA<sub>2</sub> within the host component is fundamental to the process of tumor angiogenesis and tumorigenesis and holds many implications for the use of cPLA<sub>2</sub> inhibitors in the clinic. In irradiated ovarian carcinoma cells, treatment with the cPLA<sub>2</sub> inhibitor, arachidonyltrifluoromethyl ketone (AACOCF<sub>3</sub>) (Table I), prevented the activation of Akt and enhanced cell death (13). The translational relevance of these findings was further supported by *in vivo* data showing that in a mouse model of ovarian carcinoma, the combined treatment of AACOCF<sub>3</sub> and radiation, significantly delayed tumor growth by 10 days, compared to mice that received radiation alone (13). On the whole, these reports establish a principal function for cPLA<sub>2</sub> in tumor resistance to ionizing radiation.

*Lysophosphatidic acid, autotaxin and lysophosphatidic acid receptors.* As mentioned previously, active cPLA<sub>2</sub> produces LPC, a lipid second messenger that activates a wide range of cell types within the vascular system and which can regulate a variety of biological functions including cytokine synthesis, endothelial growth factor expression, and chemotaxis (14, 15, 17-19). Alternatively, LPC can also be subsequently converted into lysophosphatidic acid (LPA) by autotaxin (20, 21). Reports from a variety of laboratories

have shown that autotaxin, LPA, and LPA receptors are often overexpressed in numerous cancer types including non-small cell lung cancer (NSCLC) (22), glioblastoma (23) and ovarian carcinoma (24, 25). Excessively high levels of these lipid mediators are frequently associated with tumor invasiveness, metastatic potential, and angiogenesis (22, 23, 26-31). Our screening experiments for the expression profile of autotaxin and LPA receptors in vascular endothelial, ovarian and lung cancer cell lines demonstrated that although autotaxin was secreted at detectable levels in human umbilical vein endothelial cells (HUVEC) and murine tumor vascular endothelial cells, the maximal expression of the enzyme was observed in conditioned medium from cancer cells (13, 32). LPA production was also significantly increased in irradiated A2780 ovarian cancer cells (13) and co-cultures of HUVEC and A549 (32). This suggests that in response to ionizing radiation, LPC is hydrolyzed to LPA by autotaxin, secreted by tumor cells. Since we had previously shown that ionizing radiation can activate pro-survival signaling, we investigated the effects of tumor-secreted factors on extracellular signal-regulated kinases 1 and 2 (ERK1/2) phosphorylation in co-cultures of HUVEC and A549 cells. Remarkably, in HUVEC treated with conditioned medium from A549 cells, irradiation with 3 Gy substantially increased ERK1/2 phosphorylation, further emphasizing the involvement of tumor-vascular endothelial communication in radiation resistance (32).

As a major regulator of biological activities, LPA can exert its effects through specific LPA receptors (LPA1-4) and G protein-coupled receptors belonging to the endothelial differentiation gene (EDG) family (26, 27, 31). Following treatment of vascular endothelial-NSCLC co-cultures with the autotaxin inhibitor and pan-LPA receptor antagonist, *a*-bromomethylene phosphonate LPA (BrP-LPA) (Table I), we observed differential effects on the invasiveness of vascular endothelial cells (32). When HUVEC were co-cultured with human large cell lung cancer cells H460, which express low levels of autotaxin and LPA<sub>2</sub>/EDG-4, treatment with BrP-LPA, prior to irradiation, had no effect on the invasion of HUVEC. By contrast, HUVEC co-cultured with A549 cells, which express higher levels of autotaxin and LPA<sub>2</sub>/EDG-4, exhibited significantly reduced invasion following treatment with BrP-LPA and 3 Gy irradiation (32). These findings were in accordance with results from glioblastoma and murine brain vascular endothelial cells (27). Blockade of LPA signaling by BrP-LPA attenuated clonogenic survival and tubule formation in irradiated mouse brain microvascular (bEnd.3) cells and GL261 glioma cells. When bEnd.3 cells were grown in co-culture with GL261 cells, treatment with BrP-LPA prior to irradiation with 3 Gy reduced the activation of Akt pro-survival signaling in both cell types (27). Evidence from the observed phenotype *in vitro* was strengthened by *in vivo* studies that demonstrated a

significant tumor growth delay of 6.8 days (relative to irradiation alone) in glioma-bearing mice that received a combination of BrP-LPA and irradiation (27). Collectively, these data illustrate key roles for autotaxin and the cPLA<sub>2</sub>-mediated production of LPC and LPA in radioresistance. As a result, inhibition of this signal transduction pathway may improve the tumor response to radiation therapy in NSCLC, glioblastoma and ovarian carcinoma.

*Insulin-like growth factor 1 receptor.* Given the relevance of molecular communication networks to the cellular viability of irradiated tissues, some studies are now focusing on the prognostic significance of key signaling molecules. One such example is the expression of insulin-like growth factor 1 receptor (IGF-1R). IGF-1R is a widely distributed cell membrane receptor that triggers cell growth and survival through the phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signaling pathways (33). Experiments using mouse embryonic fibroblasts revealed that cells without IGF-1R expression displayed higher levels of radiation-induced apoptosis (33, 34). Conversely, irradiated cell lines that overexpressed IGF-1R were resistant to cell death (33-35). In addition to alleviating radiosensitivity, IGF-1R also has an established link to the maintenance of cellular transformation (33, 36). *In vitro* studies of estrogen receptor (ER)-positive breast cancer cells demonstrated increased IGF-1R levels (33, 37). Furthermore, in patients with breast cancer who experienced local relapse after irradiation, immunohistochemical staining of tissue specimens detected high levels of IGF-1R expression in recurrent tumors (33, 35). Similar results were observed in patients with cervical cancer treated with chemoradiotherapy (33, 38). Out of 60 patients, 50 exhibited complete response six to nine weeks after the cessation of treatment. To determine the effects of IGF-1R on long-term local control and survival in complete responders, tissue samples were collected and stained for IGF-1R protein. The patients were then monitored periodically for evidence of tumor recurrence. Low IGF-1R levels were observed in tumor sections from 19 complete responders. Moreover, 100% of the patients with low IGF-1R staining remained free of local, distant, and death-related disease at five years post-treatment, compared to only 19% of those patients bearing high levels of IGF-1R (33, 38). These findings support the hypothesis that overexpression of IGF-1R is a predictive factor for increased recurrence and inadequate long-term control of local disease (33).

Comparable results were also published in a study of nasopharyngeal cancer (33, 39). Prior to chemoradiation therapy, IGF-1R immunohistochemical staining was performed on tissue specimens collected during biopsy revealing IGF-1R overexpression in 42 out of 75 tissues. Interestingly, overexpression of epidermal growth factor receptor (EGFR) was also present, and there was a positive

correlation between increased EGFR/IGF-1R levels and recurrence (33, 39). Patients with nasopharyngeal cancer with EGFR/IGF-1R-positive tumors displayed significantly reduced 5-year survival rates (33, 39).

Due to the apparent clinical significance of IGF-1R, many groups are focused on developing an effective blockade of IGF-1R signaling. Monoclonal antibodies to IGF-1R have shown promising results in recent applications (33, 40). When used in combination with ionizing radiation, the inhibition of IGF-1R significantly enhanced G<sub>2</sub> accumulation and cell death in multiple NSCLC cell lines (33, 40). In head and neck cancer cell lines, the combined treatment with the anti-IGF-1R monoclonal antibody A12 (Table I) and radiation resulted in apoptosis and necrosis (33, 41). Another IGF-1R antibody, CP-751,871 (Table I), also demonstrated a radiosensitizing effect in NSCLC lines (42). In addition, treatment with both CP-751,871 and fractionated radiotherapy delayed NSCLC tumor growth *in vivo* by 27.2 days compared to 6.4 days with radiation alone (42). Hence, the inhibitory blockade of IGF-1R may potentially enhance the tumor response to radiation therapy in the clinic.

As stated earlier, IGF-1R overexpression is often accompanied by the elevated expression of other receptor tyrosine kinases (RTKs) such as EGFR (33, 39). This association between two powerful signaling kinases makes dual inhibition an attractive therapeutic strategy. In patient-derived glioblastoma cell lines, the antibody-mediated inhibition of IGF-1R and EGFR with AG1024 (Table I) and AG1478, respectively, resulted in elevated radiation-induced apoptosis (43). IGF-1R can also compensate for loss of EGFR signaling, thus, providing further validation for a dual targeting approach (43).

**Nutlins and p53 regulation.** Another avenue of strategic signaling inhibition for tumor radiosensitization involves targeting protein-protein interactions. For many years, the field of cancer research has recognized the tumor suppressor protein p53 as being the gatekeeper for several stress response pathways (44-48). p53 participates in a complex network of molecular events that result in biological responses ranging from cell cycle arrest, apoptosis, and senescence to migration, angiogenesis, and DNA repair (6, 44-49). Extensive investigative analysis has shown that p53 activity is regulated at multiple levels, including interaction with murine double minute 2 (MDM2) protein (6, 50, 51). p53 binds to the promoter of the *MDM2* gene and transcriptionally activates MDM2 protein expression. MDM2 can then bind to p53 and block its interaction with the basal transcriptional machinery. As a result, the ability of p53 to induce gene expression is compromised (6, 52, 53). Additionally, MDM2 is the major E3 ubiquitin ligase responsible for p53 ubiquitination and subsequent proteosomal degradation (6, 50, 51, 54-57).

Multiple studies have shown that ionizing radiation induces p53-dependent *MDM2* gene transcription (6, 58, 59). This inhibits p53 transcriptional activity and favors the nuclear export of p53 to the cytoplasm where it can no longer function as a transcriptional factor (6, 58-61). Through the attenuation of p53-mediated cell cycle arrest and apoptosis in response to DNA damage, MDM2 limits the effects of irradiation on tumor cells (6). Consequently, targeting the auto-regulatory feedback loop by which MDM2 and p53 mutually control one another could be an efficient radiosensitization strategy.

Currently, the most popular approach to reducing the p53/MDM2 interaction in irradiated cells is to combine radiation with *cis*-imidazole derivatives known as nutlins (Table I) (6, 62-64). Potent and selective small molecules, nutlins bind to the hydrophobic p53-binding pocket on MDM2, thus antagonizing the p53/MDM2 interaction (6, 64, 65). Data from seven patient-derived laryngeal carcinoma cell lines revealed that cells expressing wild-type p53 were significantly more sensitive to radiation, following treatment with nutlin-3 (66). Similar effects were observed in prostate cancer cell lines treated with nutlin-3 and ionizing radiation; only cell lines expressing wild-type p53 demonstrated radiosensitization (6, 67). These data suggest that nutlins may not be effective in cancers with p53 inactivation. To test the hypothesis that loss of functional p53 may limit the effectiveness of nutlins in radiosensitization, Supio *et al.* evaluated the response to nutlin-3 in irradiated prostate cancer lines of different p53 status (63). Interestingly, under low levels of oxygen, nutlin-3 improved the radiosensitivity of prostate cancer cells in a p53-independent manner (63). Such findings provide evidence that p53 may not be required for the nutlin-mediated radiosensitization of hypoxic cells. Furthermore, nutlins can also inhibit vascular endothelial growth factor (VEGF) production and reduce tubule formation in irradiated vascular endothelial cells (62, 68-70). This direct antiangiogenic effect classifies nutlins as valid therapeutic options for the radiosensitization of tumors, regardless of p53 status. One potential concern, however, is that nutlins may increase radiation-induced toxicity in normal cells and tissues. Fortunately, the most recent attempts to assess this risk have provided preliminary reassurance. In a study using nude mice, the administration of nutlins as a single treatment was well tolerated and did not result in severe cytotoxicity (6, 64, 65). Moreover, the genetically modified reduction in MDM2 and subsequent up-regulation of p53 only resulted in mild toxicity to the highly radiosensitive hematopoietic system and small intestine (6, 71, 72). Based on these studies, researchers are cautiously optimistic that combined regimens of nutlins and radiation may significantly improve tumor control.

## Cancer Stem Cells

Perhaps one of the most challenging areas of radiation biology is determining the cell populations that are responsible for persistent growth and tumor recurrence. One widely accepted model involves the contribution of cancer stem cells (CSCs). The American Association for Cancer Research defines CSCs as a finite subpopulation of cells within the tumor that have the exclusive ability of self-renewal and tumor maintenance. According to this definition, only CSCs can expand the CSC reservoir and differentiate into the heterogeneous lineages of cancer cells that comprise the tumor (1, 73, 74). Not surprisingly, evaluating cancer from this developmental perspective has led to a variety of novel targeting strategies in radiotherapy.

The starting point for determining radiosensitization of CSCs involves combining recognized CSC surface markers with known radiobiological endpoints (1). One putative CSC biomarker is CD133. A 92- to 110-kDa membrane glycoprotein, CD133 is frequently used to enrich for stem-like cells in glioblastoma multiforme (GBM) (1). In a series of experiments, fractionated radiotherapy resulted in enrichment of the CD133+ cell fraction, as well as reduced radiation-induced apoptosis in GBM (1, 75). CD133+ cells exhibited enhanced DNA repair and tumorigenicity (1, 75). Moreover, CD133+ medulloblastoma cells were resistant to radiation in comparison with CD133- cells (1, 76). CSC markers are also frequently studied in breast carcinoma (77-79). The MDA-MB-231 breast cancer cell line has been sorted for two breast CSC markers, CD24 and epithelial-specific antigen (ESA) (80). CD24 expression is important during embryonic development and for the maturation of hematopoietic cells (1). Although CD24 expression is not as relevant for identifying stem cell populations in other tumors, the lack of CD24 in breast cancer is implicated in invasiveness, metastasis, and stemness of CSCs (77-79). Following fractionation, CD24-/ESA+ MDA-MB-231 cells contained an increased proportion of mammospheres and exhibited enhanced xenograft formation compared to unsorted cells (80). Furthermore, this subfraction displayed a radioresistant phenotype characterized by reduced foci of histone H2AX phosphorylated on serine 139 ( $\gamma$ -H2AX); formation of  $\gamma$ -H2AX foci is often an indicator of DNA double-strand breaks in response to genotoxic stress (80). Additional confirmation for CSC-dependent tumor radioresistance was obtained from studies using early passage, patient-derived primary breast CSCs in a xenograft model (5). In UM2 xenografts that were estrogen- and progesterone receptor-positive, a single dose radiation treatment of 8 Gy resulted in the enrichment of a lineage-negative CD44+/CD24- subpopulation and increased mammosphere formation capability (5). It is necessary to mention, however, that the hormone-negative MC1 breast

cancer line experienced a rapid and progressive decline in the proportion of CSCs in irradiated tumors (5).

The identification of such discrepancies only highlights the need for a more discernible approach to evaluate the contribution of the CSCs subfraction to radioresistance. One dilemma that often presents itself within the field is the lack of consistency among CSC model selection (1). Primary patient-derived cells are considered to be the most appropriate model system, but primary cancer cells are generally difficult to obtain. Together with the limited lifespan of the cultures, this CSC model can make data replication quite challenging.

To circumvent these obstacles, many laboratories rely solely on established cell lines as their source for CSC. Other potential hurdles involve the evaluation of the CSC response to radiation. For example, the predominant mechanisms of radiation-induced cell death are mitotic catastrophe (81-83) and senescence (84-86). Despite this fact, many studies focus solely on apoptosis assays when evaluating the survival of irradiated CSCs (1, 87). Likewise, the measurement of DNA damage, using residual  $\gamma$ -H2AX foci, is not always representative of CSC clonogenicity (80, 87). This incongruity is especially apparent in pancreatic cancer cell lines (80). Compared to unsorted cells, CD24+ESA+ Panc-1 and PSN-1 cell lines exhibited a 40% and 61% reduction, respectively, in residual  $\gamma$ -H2AX foci, but displayed no heightened radioresistance as determined by clonogenic assay (80). Other inconsistencies between studies that describe the radiosensitivity of CSCs could be due to tumor-specific effects of CSC marker profiles. The expression or positivity of CSC markers differs greatly between tumor types (*e.g.* breast CSCs are often CD24- while pancreatic CSCs are CD24+) (1).

Currently, experts in the field of stem cell research are focused on the identification of novel, yet stable and discriminatory CSC markers to be used to enhance standard radiation therapy (Figure 1). One functional marker of interest is aldehyde dehydrogenase 1 (ALDH1) (Table I). ALDH1 is expressed at very high levels in the liver and kidneys (1, 88, 89). When active, this enzyme oxidizes intracellular aldehydes to carboxylic acids and serves as a cytosolic detoxifying system (88, 89). Recently, ALDH1 activity has become a tool for CSC enrichment and is correlated with poor prognosis in breast, pancreatic, NSCLC, prostate, and head and neck squamous cell cancers (89-91). The role of ALDH1 as a prognostic factor strengthens its appeal as a possible component of the radiation response. Accordingly, two breast cancer cell lines were sorted on the basis of CD44 expression and ALDH1 activity and were shown to exhibit varying degrees of radiosensitivity (1, 92). ALDH<sup>high</sup> CD44+ cells were significantly more radioresistant than their ALDH<sup>low</sup> CD44- counterparts. Consequently, inhibition of ALDH1 resulted in radiosensitization of the ALDH<sup>high</sup> CD44+ population (1, 92).

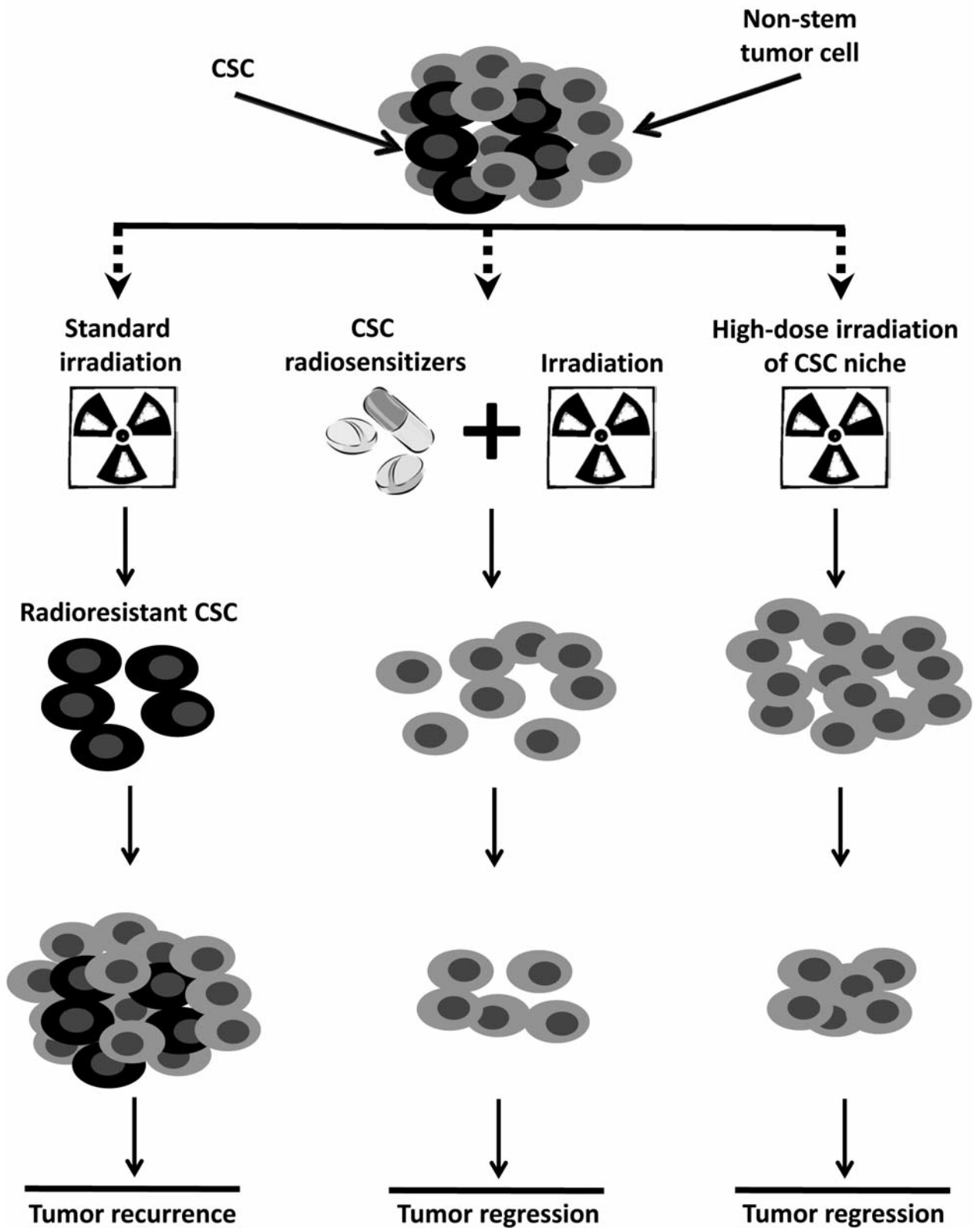


Figure 1. Targeting cancer stem cells (CSC) with ionizing radiation. Standard radiation therapy fails to eradicate CSCs. This often leads to tumor repopulation and disease progression. High-dose irradiation of the CSC niche or administration of CSC-specific radiosensitizing agents prior to irradiation may reduce radioresistant subpopulations and lead to overall tumor regression.

Aside from the development of novel CSC biomarkers, the other promising therapeutic strategy in CSC radiation biology is the targeting of specific niches that support the stem cell phenotype (Figure 1). A retrospective clinical study revealed that the subventricular zone (SVZ) may also serve as a niche for brain tumor stem cells (93). Glioma patients who received a higher dose of radiation to the SVZ lived significantly longer than those who were treated with lower doses (93). Since the SVZ is known to harbor neural stem cells, it may also provide a safe haven for glioma stem cells. Pending further validation results from the retrospective study, this suggests that the SVZ may be a critical target for radiation therapy in patients with brain tumor.

### Radiation-induced Immunotherapy

Apart from deciphering signal transduction pathways and targeting subpopulations of tumor cells, researchers are now attempting to augment the efficacy of radiation therapy by harnessing the power of the immune system. Ongoing studies of the immunological response to radiation revealed that irradiated cells present an altered antigenic peptide repertoire and elevated expression of major histocompatibility complex (MHC) class I (94-101). Furthermore, radiation evokes active secretion of cytokines that stimulate CD8+ cytotoxic T-cells and attract dendritic antigen-presenting cells (94-101). Together, these signals collaborate to launch an antitumor immune response.

Efforts to exploit this radiation-inducible mechanism have yielded favorable pre-clinical results. Using radioresistant B16SIY murine melanoma, Meng *et al.* treated cells with a combination of ionizing radiation and veliparib (Table I), an inhibitor of poly(ADP-ribose) polymerase (PARP) (99). This combined treatment led to persistent DNA damage and accelerated senescence characterized by persistent  $\gamma$ -H2AX foci and  $\beta$ -galactosidase staining, respectively (99). To investigate the influence of adaptive immunity, cells were treated with veliparib plus radiation and were implanted on the right hind limb of C57/BL6 mice. As an immune challenge, untreated B16SIY cells were injected in both hind limbs seven days later. The tumor take rate for control mice (no vaccinated cells) was 100% within two weeks of injection (99). In comparison, 80% of mice inoculated with senescent cells prior to challenge failed to form tumors. In order to form tumors, senescent B16SIY cells required the presence of functional dendritic cells and CD8+ T-cells (99). These findings infer that cell-based cancer vaccines may extend the value of radiotherapy to prevent not only local recurrence, but also distant metastasis. Thus, this approach could be utilized as a novel therapeutic combination of radiation and immune response (Figure 2).

### Using Radioprotection of Normal Cells to Enhance Radiation-induced Tumor Cell Death

Tumor sensitization is crucial for the potentiation of radiation therapy. In order to improve the efficacy of this powerful anticancer treatment, however, the topic of radioprotection should not be ignored (Table II). Multiple lines of evidence indicate that increasing the cumulative radiation dose by as little as 10-20% may facilitate the complete eradication of some tumors (102, 103). Unfortunately, elevated doses of ionizing radiation result in severe damage to tumor-surrounding normal tissues, especially to two of the most radiosensitive tissues – the hematopoietic system and the gastrointestinal tract (103, 104). Accordingly, if higher doses of radiation are to be utilized, healthy tissues must be protected. Currently, the only FDA-approved drug for protection of radiation-induced cytotoxicity is amifostine (Table II) (103, 104). Nevertheless, toxicity and insufficient selectivity for the protection of normal cells over tumor cells regrettably make amifostine an unacceptable candidate for the distinct protection of normal tissues.

Encouraging pre-clinical data implies that superior and increasingly selective pharmacological agents may soon replace amifostine (103, 105, 106). The development of these new agents is based on the premise that the cytotoxicity of ionizing radiation is cell-cycle dependent; cells are most radiosensitive during the G<sub>1</sub>/S transition and G<sub>2</sub>/M phase (103, 105). In light of this observation, Johnson *et al.* investigated the plausibility of cell cycle-targeting agents as potential radioprotectors (106). Cyclin-dependent kinases (CDKs) are essential for advancing each phase of the cell cycle. Not surprisingly, the deregulation of CDK activity is a common occurrence during tumorigenesis. To determine the effects of CDK inhibitors on radiation-induced cell toxicity, Johnson *et al.* treated cells with two inhibitors of CDK4/6, specifically PD0332991 and 2BrIC (Table II). In cells positive for the tumor suppressor retinoblastoma protein (pRb), PD0332991 and 2BrIC caused reversible G<sub>1</sub> arrest, also known as pharmacological quiescence (106). Cells deficient for pRb did not undergo growth arrest. Treatment with CDK4/6 inhibitors also protected pRb-positive cells from extensive radiation-induced DNA damage and cell death. *In vivo* experiments using a genetically engineered murine model of melanoma showed that a single dose of PD0332991 four hours prior to total body irradiation produced a striking rescue of weight loss and mortality, without any change in tumor survival (106). Such reports indicate that CDK4/6 inhibition can significantly reduce radiation-associated myelosuppression, without protecting CDK4/6-independent tumor cells (103, 106). Consistent with evidence that tumor cell radioprotection by CDK4/6 inhibitors is possible only in the small population of pRb<sup>+</sup>/p53<sup>WT</sup> tumors, compounds like PD0332991 may be widely-used to protect hematopoietic

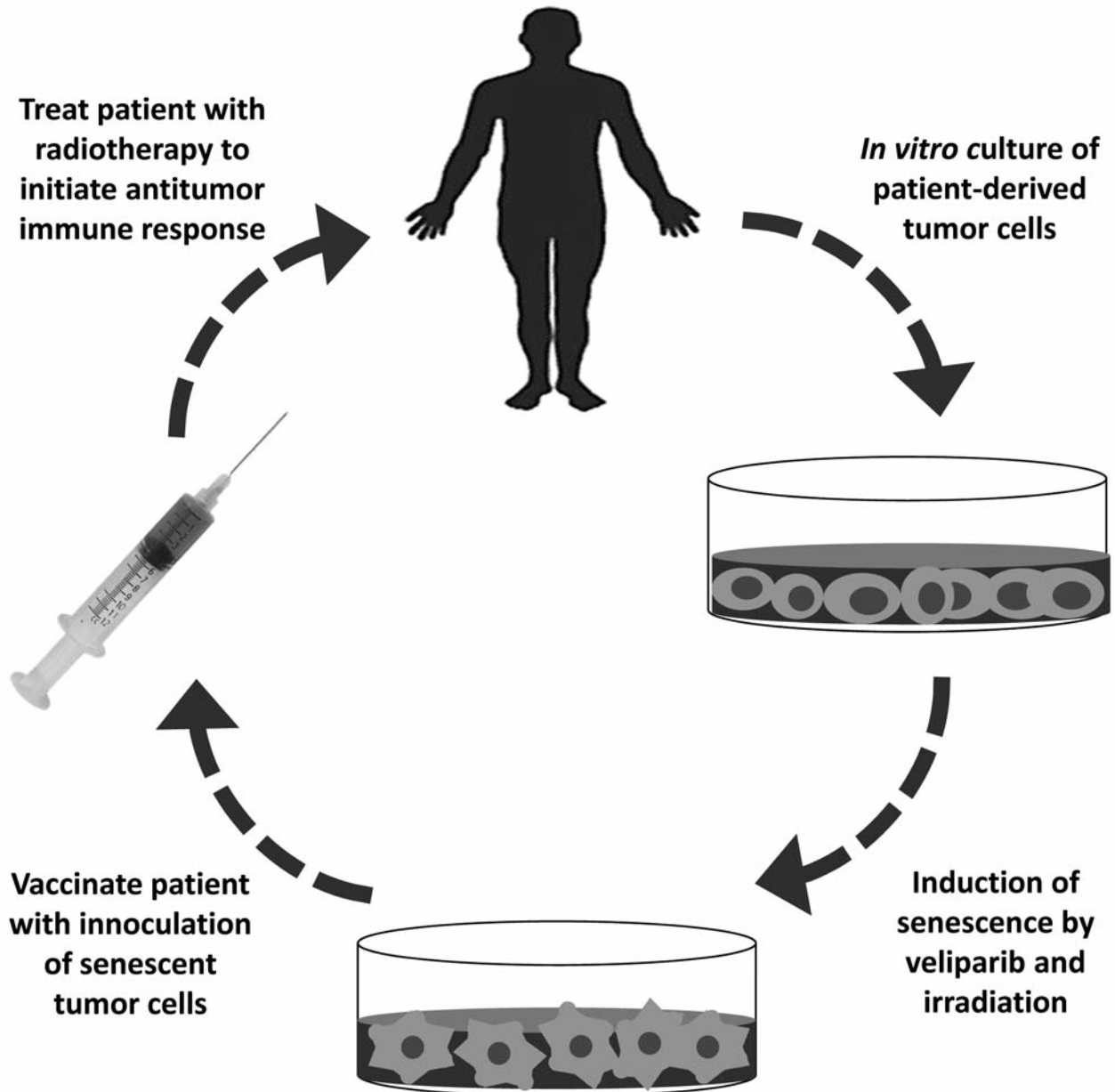


Figure 2. Using radiotherapy to induce an antitumor immune response. After treatment with veliparib and ionizing radiation, senescent cells from patient-derived tumors can be used to induce an antitumor immune response. This radiotherapy-mediated tumor vaccine may lead to radiosensitization of the primary tumor, as well as of metastatic disease.

progenitors from genotoxic damage, without compromising tumor cell kill (103, 106).

In our study of neurocognitive dysfunction resulting from cranial irradiation, we demonstrated that glycogen synthase kinase 3 $\beta$  (GSK-3 $\beta$ ) is required for radiation-induced hippocampal neuronal apoptosis (107, 108). Inhibition of GSK-3 $\beta$  with lithium or small molecules SB216763 or SB415286 (Table II), prior to irradiation significantly

attenuated radiation-induced apoptosis in hippocampal neurons in cell cultures and in mouse models. Interestingly, use of the same inhibitors did not affect radiation-induced death of glioma or medulloblastoma cell cultures (107, 108). Further investigations demonstrated that ionizing radiation triggers distinct GSK-3 $\beta$ -dependent signaling in normal and cancer cells, allowing for specific radioprotection of normal tissue without affecting the tumors (107-109).



Table II. Current radioprotectors and their respective targets.

Therapeutic agent	Target	Treated cells or tumors	Stage of clinical development	References
Amifostine	Reactive metabolites of radiation, cisplatin and alkylating agents	Normal tissues with high levels of alkaline phosphatase	FDA-approved since 1995	(103, 105, 106)
PD0332991	Cyclin-dependent kinases 4/6 (CDK4/6)	Normal tissues	Pre-clinical for radioprotection	(106)
		Melanoma, glioma, ovarian carcinoma, colorectal cancer, breast cancer	Phase I/II clinical trials for antitumor effects only	(106)
2BrIC	Cyclin-dependent kinases 4/6 (CDK4/6)	Melanoma	Pre-clinical	(106)
Lithium	Glycogen synthase kinase 3 $\beta$ (GSK-3 $\beta$ )	Hippocampal neurons	Phase I/II clinical trials for radioprotection	(107)
SB216763	Glycogen synthase kinase 3 $\beta$ (GSK-3 $\beta$ )	Hippocampal neurons and intestinal epithelial crypt cells	Pre-clinical	(108-110)
SB415286	Glycogen synthase kinase 3 $\beta$ (GSK-3 $\beta$ )	Hippocampal neurons and intestinal epithelial crypt cells	Pre-clinical	(108-110)
Pyridoxamine	Reactive oxygen and carbonyl species	Intestinal epithelial crypt cells	Pre-clinical for radioprotection	(111)
			Phase I/II clinical trials for non-irradiation	(113, 114)

We also demonstrated that small-molecule inhibitors of GSK-3 $\beta$  protect mouse gastrointestinal tract from radiation-induced damage and improve survival of mice treated with the lethal dose of total body irradiation (110). Similar effects were observed with the free radical-scavenger pyridoxamine (Table II) (111). Moreover, pyridoxamine was more effective at protecting from radiation-induced apoptosis than amifostine; being at the same time well-tolerated, with no significant treatment-related adverse effects (112). In clinical trials, pyridoxamine ameliorated diabetic renal injury, thus receiving approval from the US Food and Drug Administration (FDA) for a Phase 3 trial (113, 114). These emerging radioprotectors present an alternative strategy for tumor radiosensitization, as well as for reduction of deleterious consequences of normal tissue irradiation, and would thereby improve quality of life during radiation therapy.

## Conclusion

Radiation therapy offers significant clinical benefit to patients with advanced cancer, but long-term tumor control is often compromised by local and distant failures. Previous therapeutic advances have provided some improvement, however, many of these strategic developments result in mechanistic resistance or intolerable cytotoxicity to normal tissues. Adding further complication, the radioresistance of individual cancer cells is influenced by a wide variety of intrinsic and extrinsic factors. As such, cooperative therapeutic efforts that focus solely on tumor cells, yet fail

to recognize the importance of paracrine interactions and the tumor microenvironment, can never fully achieve the goal of enhanced treatment efficacy. Fortunately, the recent discovery of novel targets for ionizing radiation may soon enable radiation therapy to completely eradicate tumor cells without harming their non-tumorigenic counterparts. Thus, elucidation of the molecular mechanisms underlining tumor radioresistance is critical for the complete destruction of malignant tumors.

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