Towards novel radiosensitizing agents: the role of cytosolic PLA2α in combined modality cancer therapy

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Abstract

The radioresistant nature of some tumors serves as an obstacle to curative therapy for several poor-prognosis malignancies. The radiosensitivity of a cancer is dependent not only on the intrinsic ability of tumor cells to recover from radiation-induced damage, but also the ability of stromal elements (e.g., vasculature) in the tumor microenvironment to survive and continue proliferating in the face of ionizing radiation. In this regard, it is important to understand the initial events activating radiation-induced signal transduction pathways. Among these events is the activation of cytosolic phospholipase A2α and the subsequent production of the lipid second messengers. These events occur within minutes following exposure to ionizing radiation, and have been shown to enhance cell viability through a number of prosurvival signaling pathways. Furthermore, inhibition of cytosolic phospholipase A2α has now been shown to reduce the viability of endothelial cells in culture after exposure to ionizing radiation, as well as slowing the growth of tumors in animal models of cancer.

Radiotherapy in difficult to treat malignancies

Radiation therapy (RT) remains an integral part of modern cancer management for both benign and malignant tumors. More than 50% of newly diagnosed cancer patients worldwide receive RT (alone or in combination with chemotherapy or surgery) at some point during the course of their treatment [1]. Compared with both surgery and chemotherapy, RT is unique in that it is generally noninvasive and devoid of intense systemic toxicity. It is, therefore, an integral component of organ preserving and adjuvant treatment strategies for primary tumors and palliative treatment strategies for advanced and metastatic tumors.

In the past decade, there have been substantial improvements in radiation treatment outcomes attributable to advances in clinical, physics and biology research [2]. These advances have permitted more cancers to be treated with higher and more tumoricidal doses of radiation with curative intent [3,4]. Despite these improvements in therapeutic regimens, local recurrence of some malignancies, including lung cancer and glioblastoma multiforme, remain persistant problems [5,6]. These tumor types are highly angiogenic and resistant to radiation [7,8]. Despite aggressive treatment, most patients with unresectable glioblastoma

multiforme have a median survival of approximately 1 year [7–9], while patients with unresectable non-small-cell lung cancer (NSCLC) have a similarly poor prognosis with median survivals of approximately 18 months [10,11]. There is, therefore, an ongoing need in RT to develop new approaches for the treatment of these difficult-to-cure cancers.

An improved understanding of the molecular mechanisms contributing to tumor radioresistance has led to the conclusion that ionizing radiation (IR) not only damages nuclear DNA, but also activates a complex series of signaling cascades within the plasma membrane and cytosol [12,13]. While the exact function or purpose of some of these pathway activation events remains unclear, several are likely to contribute to the radioresistance of tumors. As a result, a number of drug-discovery targets have been identified with numerous attempts to test modulatory compounds in various clinical and preclincal studies of radiosensitization [14,15].

Need for novel radiosensitizers: past, present & future attempts to improve the radiation therapeutic ratio

Early efforts that endeavored to discover **radio-sensitizing** agents concentrated on hypoxia-sensitizing compounds. Tumor hypoxia has been well recognized as a major factor contributing to radioresistance, since histologic evidence for tumor hypoxia in human neoplasms was first reported in 1955 [16]. Studies with direct measurement by microelectrodes have revealed heterogeneity in intratumoral oxygen concentrations and low oxygen concentrations are associated with poor local–regional control by RT [17]. These findings, coupled with the result of nuclear imaging studies employing radiolabelled imidazoles, have provided strong evidence for the existence of tumor hypoxia that influences RT treatment outcomes [18]. Since the mid-1970s, clinical research into overcoming tumor hypoxia has mainly focused on the use of nitroimidazoles as hypoxic cell sensitizers. Unfortunately, the results from numerous major clinical trials were largely discouraging [19–21]. Second-generation agents did not fare much better with the European etanidazole (ETA) trial demonstrating no advantage of adding the drug to RT [22], and the Radiation Therapy Oncology Group ETA trial failing to show advantage of using the drug on the trial population as a whole [23].

Given the unimpressive clinical results with nitroimidazole-mediated radiosensitization, hypoxic cytotoxins, such as tirapazamine, were then offered as the next potential hope for targeting radioresistant cells. Tirapazamine is a bioreductive agent, which by undergoing one-electron reduction in hypoxic conditions, forms cytotoxic free radicals that produce DNA strand breaks, leading to cell death. *In vitro* and *in vivo* laboratory studies demonstrate that tirapazamine is 40- to 150-times more toxic to cells under hypoxic conditions when compared with oxygenated conditions. Furthermore, tirapazamine is superior to ETA in enhancing fractionated irradiation in mouse squamous cell carcinoma (SCCVII) and other tumor types [24,25]. Despite these impressive preclinical results, several Phase III trials have failed to demonstrate any survival benefit from the addition of tirapazamine to chemotherapy or RT in NSCLC or head and neck squamous cell carcinomas (HNSCCs) [26].

While the results of these studies have generated a slightly 'nihilistic' view with regards to the development and implementation of an effective radiosensitizing drug, clinical oncology is not without widespread use of other agents as radiosensitizers. Indeed, there is little question that the combination of certain cytotoxic chemotherapeutic agents with RT has been, historically and currently, the most clinically efficacious and widely utilized means of cancer radiosensitization [27,28]. This approach has led to improved treatment results in patients with advanced solid tumors. In particular, the concurrent application of both

modalities has proven effective and resulted in lower recurrence rates and improved survival in several tumor types, such as HNSCC [29], advanced-stage lung [30] and cervical cancer [31]. More recently, it has been shown that even in notoriously therapy- resistant tumors, such as gastric cancer and glioblastoma, the addition of chemotherapy to radiation improves survival [32,33].

Although the successful combination of cytotoxic chemotherapy and radiation is undeniable, both the acute and late normal tissue toxicity of these combined-modality regimens often remains problematic. For example, in a seminal Phase III trial that established the survival superiority of chemotherapy combined with RT over RT alone in head and neck cancer, patients who received chemotherapy and radiation experienced 89% grade 3–5 toxicity, compared with 52% in the radiation alone group [29]. Therefore, owing to the nonspecificity of traditional chemotherapeutic agents, tumor control efficacy has largely been limited by normal tissue toxicity. This naturally led to the investigation and development of molecularly targeted agents that, theoretically, would have less severe normal tissue side effects owing to their relative tumor selectivity. An example of an agent that has been clinically successful in this regard is cetuximab, an EGF receptor (EGFR) inhibitor. In a landmark Phase III trial, cetuximab significantly improved locoregional control and overall survival in HNSCC when combined with radiotherapy, compared with radiotherapy alone. Importantly, this benefit was achieved with little increase in toxicity (other than acneiform rash) over that associated with radiotherapy alone [34].

Several other targeted agents have since proven to be clinically useful, either as part of a combined modality regimen or as monotherapy for a variety of tumor types. Besides EGFR inhibitors, other examples of targeted agents with proven and US FDA-approved clinical utility include trastuzumab in HER-2/neu overexpressed breast cancer [35], gefitinib and other small-molecule tyrosine kinase inhibitors in well-defined subsets of patients with NSCLC [36] and vascular endothelial growth factor inhibitors such as bevacizumab [37]. Most of these agents are either approved or are in advanced clinical trials as radiosensitizers.

Recent advances in the understanding of how the tumor microenvironment responds to therapeutic intervention have proven important in developing efficient molecular targeted pharmacological agents [38–40]. It has recently been demonstrated, for example, that activation of **cytosolic phospholipase A2α** (cPLA2α), an enzyme involved in the formation of lipid second messengers, stimulates proliferation of vascular endothelial cells and promotes the formation of a functional tumor vascular network, thereby contributing to cancer progression [41,42]. Upon activation, cPLA2α translocates from the cytosol to the cell membrane [42,43], where it specifically cleaves phosphatidylcholine [44–46] to produce the oncologicaly important bioactive lipids **lysophosphatidylcholine** (LPC), **lysophosphatidic acid** (LPA) and arachidonic acid (AA) [41–42].

Targeting early mediators of radiation response: cPLA2α

The early molecular events that trigger the biological response to IR are initiated by the hydrolysis of water. The resulting hydroxyl radicals interact with cellular components, such as DNA, resulting in DNA strand breaks and subsequent activation of well-described signal transduction pathways in the nucleus. By contrast, immediate signal transduction initiated at the cell membrane is less well characterized. While it is known that ceramide is generated in endothelial cells within minutes of exposure to large doses of radiation (20 Gy), which subsequently results in apoptosis [47,48], endothelial cell apoptosis is not induced by lower doses of radiation (2–5 Gy). In fact, there is a growing body of literature demonstrating increased viability of vascular endothelial cells in response to low doses of ionizing radiation, due to activation of prosurvival signaling pathways [49,50]. Biologically active

lipids and proteins, such as phospholipases, lipid kinases and phosphatases, which regulate the production of lipid second messengers, are now thought to initiate prosurvival signal transduction within these cells [51–54].

cPLA2α is one of the molecules that has been implicated in diverse cellular processes. Perhaps one of the most notable functions of cPLA2α is its ability to initiate the inflammatory response. Following its production by cPLA2α, AA is metabolized through the cyclooxygenase (COX) and lipoxygenase pathways to yield prostaglandins and leukotrienes, respectively [55]. In addition, dysregulation of cPLA2α and alterations in AA release have also been observed in tumor cells [56,57]. Some of the cancers associated with the overexpression of cPLA2α include NSCLC, cholangiosarcomas, esophageal cancers and cancers of the colon and small intestine [58]. In many of these cancers, cPLA2α expression is accompanied by other cancer-associated stimuli, including EGFR signaling, transforming growth factor β (TGF-β), oncogenic Ras mutations, increased COX-2 expression and abundant levels of prostaglandin E2 (PGE2) [56,57]. As the predominant prostaglandin found in human tumors, PGE2 is often associated with a poor disease prognosis. Multiple lines of evidence have shown that COX-2-derived PGE2 can induce secretion of vascular endothelial growth factor (VEGF) and stimulate the release of additional pro-angiogenic factors within ovarian, prostate and gastric tumor cell lines [57]. This facilitates the formation of the tumor vasculature and results in aggressive tumor progression.

Lysophosphatidylcholine is another lipid second messenger generated by cPLA2α [59]. Recent publications suggest an important role for LPC in endothelial cell activation, leading to increased vascular proliferation, migration, expression of adhesion molecules and inflammation. LPC stimulates proliferation in endothelial cells by transactivating the VEGF receptor 2 (VEGF-R2) and activating Akt and extracellular signal-regulated kinase (ERK)1/2 [60]. Increased levels of LPC are linked directly to cytokine and chemokine production in endothelial cells by activating mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/Akt pathways, thus regulating the chemotaxis of particular leukocyte subpopulations during inflammation [61]. LPC also displays biphasic regulation of inflammatory factors in endothelial cells, causing activation of NF-κB at low concentrations and inhibition of NF-κB at higher concentrations [62].

More relevant to tumor control, pro-survival signal transduction activated by ionizing radiation within the vascular endothelium includes PI3K/Akt and MAPK pathways [63,64]. These pathways determine cellular response and sensitivity to radiation by ultimately controlling cell metabolism, proliferation and cell death [65]. Recently, the sequence of molecular events in irradiated vascular endothelial cells, which constitute an immediate prosurvival signaling pathway activated by IR, has been defined. This pathway involves activation of cPLA2α, followed by the increased production of LPC, transactivation of VEGF-R2 and phosphorylation of Akt and ERK1/2, which can then contribute to endothelial cell viability following exposure to IR [66].

Lysophosphatidic acid is another signaling molecule with known tumorigenic and angiogenic properties that is partly regulated by cPLA2α [67,68]. LPA is generated mainly by two different pathways:

- Generation of lysophospholipids such as LPC, lysophosphatidylethanolamine and lysophosphatidylserine from membrane phospholipids by phospholipase A2 or phospholipase A1, followed by conversion of these lysophospholipids to LPA by **lysophospholipase D** (LysoPLD or autotoxin) [69];

- ■ Generation of phosphatidic acid from phosphatidylcholine by phospholipase D, followed by conversion of phosphatidic acid to LPA by specific classes of phospholipase A2 [70].

It has been demonstrated that cell-surface G-protein coupled receptors mediate the cellular effects of LPA. At least three types of G-protein coupled receptors, Edg-2/LPA1/vzg-1 [71], Edg-4/LPA2 [72] and Edg-7/LPA3 [73], which belong to the endothelial cell differentiation gene family, have been identified as specific receptors for LPA. LPA is an intercellular lipid mediator with multiple actions, particularly as an inducer of cell proliferation, migration and survival [74]. LPA acts via specific G-protein coupled receptors on the cell surface to activate a great variety of signaling pathways [74]. The physiologic response to LPA signaling depends on cellular context and initiates biological processes including neurogenesis and tumor progression [75]. LPA receptors may prove to be a molecular target for treatment of cancer [76].

Study of the conversion of LPC to LPA has brought attention to another enzyme in this signaling pathway, LysoPLD. The human LysoPLD-encoding gene, *ENPP2*, is organized in 27 exons with alternative splicing, which results in the generation of three different isoforms. The shortest form, having 863 amino acids, was initially cloned from a teratocarcinoma [77]. In addition, lung cancers, melanoma and renal cell carcinoma have a high expression of LysoPLD [78,79]. The biological outcome of LysoPLD action will depend on the local availability of its substrates, the presence of regulatory cofactors and, ultimately, the spectrum of LPA receptors expressed on nearby target cells. What is known for certain is that the major physiological substrate for LysoPLD is LPC [80] and that LysoPLD converts exogenously added LPC into LPA *in vitro* [81]. Furthermore, LysoPLD promotes tumor aggressiveness, metastasis and angiogenesis in nude mice [82] and LysoPLD-over-expressing cells are protected against apoptosis [83]. Interestingly, NSCLC cell motility is dependent on LysoPLD expression, LPC concentration and the presence of functional LPA receptors [84,85], with specific knockdown of LysoPLD leading to decreased LPA levels and reduced cell growth and viability [86]. LysoPLD also appears to have a role in maintaining the tumor microenvironment. Specifically, in an *in vivo* angiogenesis model, LysoPLD-transfected Ras-transformed NIH3T3 cells caused more prominent new blood vessel formation than was observed with control cells. In addition, LysoPLD-stimulated human endothelial cells grown on Matrigel to form tubules in a manner similar to the effects of VEGF [87]. Given that cPLA2α produces LPC within tumors following irradiation with 2 Gy [66], it is thought that radiation-induced mobilization of LPC is converted by LysoPLD to LPA. In turn, LPA stimulates angiogenesis, cancer-cell migration and vascular permeability. Thus, LysoPLD may, itself, be a promising molecular target for pharmacological intervention, since it is critically involved in tumor progression and angiogenesis.

In summary, cPLA2α is involved in regulating the levels of at least three potent lipid mediators of tumorigenesis and angiogenesis: LPC, AA and LPA. The role of AA and LPA in tumorigenesis has been studied extensively. Since there is a broad literature linking these two lipids, their turnover and signaling to cancer progression [88,89], there has been active discussion regarding the use of AA and LPA signaling pathways as molecular targets for controlling cancer and several companies are developing drugs for this purpose [90,91]. Although relatively little is known about the role of LPC in tumor progression, recent studies have shown that it is a lipidderived second messenger that triggers the downstream activation of both PI3K/Akt and MAPK/ ERK prosurvival signal transduction pathways, resulting in a higher degree of radioresistance and increased cell viability within the tumor vascular endothelium following IR [66,91–93]. To this end, efforts to develop cPLA2α

inhibitors for use in inflammatory diseases [94,95] offer a relatively facile opportunity for the exploration of this target in the radiosensitization of cancer.

Towards clinical testing: improving management of fatal malignancies

We have attempted to summarize, briefly, the preclinical and clinical data supporting the exploitation of cPLA2α as a noncytotoxic radiosensitizer in cancer therapy. Due to its association with chronic inflammation and genomic instability, the cPLA2α -signaling pathway has become an attractive target for experimental therapeutics. While most of the therapeutic interventions have been designed to inhibit the production of AA and its downstream metabolites, recent evidence suggests that effects on other products of cPLA2α activation, LPC and LPA, may also significantly contribute to tumor progression by promoting resistance to RT through lysophospholipid-mediated angiogenesis [66,91].

Ionizing radiation is frequently used in combination with surgery and chemotherapy for the clinical treatment of solid tumors. As a result, RT has significantly improved patient-survival rates in many types of cancer. In lung cancer, however, an efficacy plateau has been reached and local recurrence continues to be a problem [96,97]. Approximately 30–40% of NSCLC patients who have distinct tumors and histologically negative lymph nodes die of recurrent disease [98,99]. Thus, the development of effective, pharmacological agents is necessary to improve the outcome of RT.

In order to create more efficacious radiosensitizing agents, the response of the tumor microenvironment to IR should be considered [100]. Several studies have demonstrated that the effectiveness of radiotherapy is often diminished due to resistance of the tumor vascular endothelium [66,91]. Upon irradiation with clinically relevant doses (2–5 Gy), IR interacts with cellular membranes to initiate signal transduction. Recent experimental evidence revealed that irradiation with 3 Gy induces cPLA2α activation followed by LPC production in vascular endothelial cells. As a biologically active lysophospholipid, LPC then triggers the downstream phosphorylation of both the Akt and ERK1/2 kinases. Activation of the PI3K/Akt and MAPK pro-survival signaling pathways results in increased cellular viability and radioresistance within the tumor vasculature. Interestingly, the inhibition of cPLA2α with short hairpin RNA or inhibition of PLA2-family members with the relatively nonspecific small molecule inhibitors (AACOCF$_3$ and methyl arachidonyl fluorophosphonate), prior to irradiation with 3 Gy, prevented the formation of a functional tumor vascular network both *in vivo* and in cell culture [66,91]. Clonogenecity experiments have also demonstrated a radiosensitizing effect of these small-molecule inhibitors when combined with IR [66]. Furthermore, when administered 30 min before irradiation, AACOCF$_3$ treatment significantly delayed lung and brain tumor growth in heterotopic tumor mouse models. While it may be argued that the radiosensitizing effect of these small molecules may be due to inhibition of other PLA2-enzymes, even more compelling evidence for exploitation of cPLA2α as a cancer drug-discovery target comes from studies with cPLA2α knockout mice (cPLA2α$^{-/-}$). Recent studies have shown that these mice formed fewer tumors compared with wild-type mice in heterotopic lung-cancer tumor models. In addition, the few tumors that did grow in cPLA2α$^{-/-}$ mice displayed attenuated vascularity compared with control animals. Since destruction of the tumor vascular network enhances the treatment of cancer [101,102], radiosensitizers that target cPLA2α and lysophospholipid production may significantly improve tumor response to RT.

To this end, we have recently begun preclinical testing with a novel cPLA2α inhibitor, which has already been tested in a pharmaceutical industrysponsored trial of osteoarthritis. Specifically, we have demonstrated that the inhibitor, PLA-695 (giripladib), reduces the activation of PI3K/Akt and MAPK pro-survival signaling pathways in cultured human

umbilical vein endothelial cells after exposure to IR. In addition, *in vivo* studies have demonstrated reduced cross sectional vascular area in lung cancer tumor models treated with PLA-695 and IR, as well as significant tumor growth delay, when compared with controls [CRAFT J & HALLAHAN D, UNPUBLISHED DATA]. Although the initial clinical trial with giripladib was terminated owing to abdominal pain associated with long-term dosing [103,201], an effective radiosensitizer would only need to be tolerated during the course of RT (i.e., 3–7 weeks).

Many important questions remain with these drugs with regards to design of treatment regimens and trials. While dose can be inferred from previous preclinical and clinical pharmacokinetic data, the significance of maintaining sufficiently high concentrations of drug to cover the target between radiation fractions remains unknown. In addition, the potential utility and safety of such inhibitors in maintenance dosing after RT or potentially even as monotherapy remains an interesting corollary area of study [104–106].

Future perspective

Although past attempts to radiosensitize cancer cells with noncytotoxic agents has been clinically disappointing, the response of the tumor microenvironment to RT remains a largely untapped source of potential targets for radiosensitization. In particular, bloodvessel formation is a fundamental process that occurs during pathological tissue growth, which is already clinically modified with targeted therapies. Unfortunately, in often fatal malignancies such as lung cancer and glioblastoma, vascularization is often excessive and associated with tumor progression, even in the face of radiation, chemotherapy and selectively targeted angiogenesis inhibitors. Although many of these modalities have produced an enhanced clinical benefit, these improvements have often proved to be transitory and are generally followed by increased tumor resistance. Thus, the development of novel effective anti-angiogenic therapies for use in combination with RT could improve tumor control in treatment-resistant vascularized cancers, such as lung carcinoma and glioblastoma. Consequently, the role of cPLA2α and its associated signaling pathways in promoting radiation-induced angiogenesis and cell survival cannot be overlooked. Further efforts, including additional preclinical testing, treatment regimen design and clinical trials will likely begin in the next few years. Thereafter, it is hoped that an efficacious drug will be delivered to the clinic capable of preventing the formation and maintenance of a functional tumor vascular network when used in conjunction with radiation.

Key Terms

Radiation therapy	Use of ionizing radiation for its cytotoxic effects on malignant cells.
Radiosensitizing	Use of pharmacologic (e.g., chemotherapy) or physical (e.g., heat) means to increase the cytotoxic effects of ionizing radiation.
Cytosolic phospholipase A2 α	Enzyme responsible for the production of lysophosphatidylcholine from membrane phospholipids.
Lysophosphatidylcholine	Lipid metabolite produced by cytosolic phospholipase A2 that is thought to play a role in the cytosolic response to radiation.
Lysophosphatidic acid	Second messenger with known role in tumorigenesis and angiogenesis.

| **Lysophospholipase D** | Enzyme responsible for generation of lysophosphatidic acid. |

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Website

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Executive Summary

- Current treatment modalities, including radiation therapy, are often ineffective in curing a number of malignancies.

- Tumor radioresistance is due in part to prosurvival signaling initiated at the cell membrane of both tumor and stromal cells at the time of ionizing radiation exposure.

- Understanding of the tumor microenvironment, including the survival and continued growth of the vasculature in response to radiation, offers new drug targets for radiosensitization.

- Cytosolic phospholipase A2 α has been shown to be crucial for the development and persistence of the tumor vasculature after radiation therapy in an animal model genetically modified to lack this enzyme.

- Inhibitors of cytosolic phospholipase A2 α, including already human-tested drugs developed for other disease areas, offer the ability to rapidly translate this knowledge to clinical radiation oncology.