Tyrosine Kinase Inhibitors Protect the Salivary Gland from Radiation Damage by Inhibiting Activation of Protein Kinase C-δ

Sten M. Wie1, Elizabeth Wellberg2, Sana D. Karam3, and Mary E. Reyland1

Abstract

In patients undergoing irradiation (IR) therapy, injury to nontumor tissues can result in debilitating, and sometimes permanent, side effects. We have defined protein kinase C-δ (PKCδ) as a regulator of DNA damage–induced apoptosis and have shown that phosphorylation of PKCδ by c-Abl and c-Src activates its proapoptotic function. Here, we have explored the use of tyrosine kinase inhibitors (TKI) of c-Src and c-Abl to block activation of PKCδ for radioprotection of the salivary gland. Dasatinib, imatinib, and bosutinib all suppressed tyrosine phosphorylation of PKCδ and inhibited IR-induced apoptosis in vitro. To determine whether TKIs can provide radioprotection of salivary gland function in vivo, mice were treated with TKIs and a single or fractionated doses of irradiation. Delivery of dasatinib or imatinib within 3 hours of a single or fractionated dose of irradiation resulted in >75% protection of salivary gland function at 60 days. Continuous dosing with dasatinib extended protection to at least 5 months and correlated with histologic evidence of salivary gland acinar cell regeneration. Pretreatment with TKIs had no impact on clonogenic survival of head and neck squamous cell carcinoma (HNSCC) cells, and in mice harboring HNSCC cell–derived xenografts, combining dasatinib or imatinib with fractionated irradiation did not enhance tumor growth. Our studies indicate that TKIs may be useful clinically to protect nontumor tissue in HNC patients undergoing radiotherapy, without negatively impacting cancer treatment.

Introduction

The majority of patients diagnosed with cancer will receive irradiation (IR) therapy either alone, or in combination with surgery or chemotherapy. Despite improvements in IR delivery, damage to healthy tissues, and the associated morbidities, can significantly impact quality of life. Furthermore, IR toxicity, especially in combination with chemotherapy, can limit the course of therapy, potentially impacting tumor eradication in some patients (1).

In the oral cavity, the oral mucosa and the salivary glands are highly sensitive to IR damage. Up to 40% of patients treated with IR for head and neck carcinoma (HNC) will develop moderate to high levels of delayed xerostomia as a result of collateral damage to the salivary glands in the IR path (2). These patients typically experience a reduction in salivary production of >50% within a few weeks of commencing therapy (3). Salivary gland hypofunction, and the resultant xerostomia, is permanent and can have a significant impact on oral health and nutrition (2). Currently, the only therapeutic agent available to protect the salivary gland is the free radical scavenger, amifostine, which is not widely used due to significant toxicity (4). Thus, there is a need for the development of new therapeutic strategies that will provide selective protection of these radiosensitive normal tissues without impacting tumor cell death.

We have previously shown that PKCδ is essential for apoptosis of salivary acinar cells in vitro and in vivo (5–10). PKCδ−/− mice are protected from IR-induced damage to the salivary gland and thymus and have a delay in mammary gland involution, a process driven by apoptosis (6, 11). Likewise, salivary epithelial cells from PKCδ−/− mice are resistant to multiple apoptotic stimuli, including IR (6, 12). Our studies have defined how PKCδ is activated in the context of apoptosis and have identified critical steps in this process that can be targeted therapeutically. Specifically, we have shown that nuclear targeting of PKCδ is necessary and sufficient for epithelial cell apoptosis and that nuclear translocation requires phosphorylation of PKCδ at Y155 and Y64 by c-Abl and c-Src, respectively (5, 10, 13). Furthermore, we have shown that pretreatment of salivary acinar cells with the tyrosine kinase inhibitor (TKI) dasatinib suppresses IR-induced apoptosis in vitro and blocks phosphorylation of PKCδ at Y155 and Y64, and nuclear translocation of PKCδ in vitro (5).

As c-Abl and c-Src play fundamental roles in activating the proapoptotic function of PKCδ in IR-treated tissues, we explored using TKIs with activity against c-Abl and/or c-Src for radioprotection of the salivary gland in vivo. Here, we report that TKIs provide robust and durable protection of salivary gland function in a mouse head and neck IR model. Our studies suggest that TKIs may be useful clinically to protect nontumor tissue in HNC patients undergoing IR without negatively impacting cancer therapy.
**Materials and Methods**

**Cell culture**

The ParC5 cell line has been described previously (14). Head and neck squamous cell carcinoma (HNSCC) cell lines (UMSCC19, Cal27, and FaDu) were cultured in DMEM/high glucose medium (Thermo Fisher Scientific, #SH30243.02) supplemented with 10% FBS (Sigma, #F2442). Cal27 and UMSCC19 cells were obtained in 2012 from Dr. Lynn Heasley (University of Colorado Anschutz Medical Campus, Aurora, CO), and FaDu cells were obtained in 2012 from Dr. Shi-Long Lu (University of Colorado Anschutz Medical Campus). Cell line profiling for authentication and mycoplasma testing was done at the DNA Sequencing Core at University of Colorado Anschutz Medical Campus using the AmpFLSTR Identifiler PCR Amplification Kit from Applied Biosystems. Cells used in these experiments were within 10 passages of testing. For some experiments, cells were treated with dasatinib (0.5–50 nmol/L; University of Colorado Hospital Pharmacy), imatinib (0.1–50 μmol/L; University of Colorado Hospital Pharmacy), or bosutinib (0.1–10 μmol/L; Selleckchem, #SKI-606) 30 minutes prior to IR or treatment with H2O2 (Thermo Fisher Scientific, #H325-500). Cells were irradiated using a Cesium-137 source.

**In vivo head and neck IR**

Cal27 and FaDu cells were obtained in 2012 from Dr. Lynn Heasley (University of Colorado Anschutz Medical Campus, Aurora, CO), or bosutinib (0.1–50 nmol/L; University of Colorado Hospital Pharmacy), or imatinib (0.1–50 μmol/L; University of Colorado Hospital Pharmacy), or bosutinib (0.1–10 μmol/L; Selleckchem, #SKI-606) 30 minutes prior to IR or treatment with H2O2 (Thermo Fisher Scientific, #H325-500). Cells were irradiated using a Cesium-137 source.

**Analysis of salivary glands**

For tissue analysis, salivary glands were formalin fixed, paraffin embedded, and sectioned at 5 μm. Tissue sections were stained with hematoxylin and eosin (H&E) and aquaporin 5 (AQP5) to detect by IHC (Abcam, #ab78486). Stained slides were archived and analyzed using the Aperio Digital Pathology System and ImageScope software (Leica Biosystems). For AQP5, the number of positive stained pixels was quantified using the positive pixel algorithm in three representative sections, cut 50 μm apart, of both full submandibular salivary glands from 2 mice per treatment group, for a total of six measurements per group.

**Statistical analysis**

Data were analyzed in Excel or in GraphPad Prism 6. Shapiro–Wilk tests for normalcy were applied, and if data were determined not to be normally distributed, nonparametric analyses were performed. Otherwise, parametric statistical analyses were performed.
analysis was performed (t tests, ANOVA). Graphical data are presented as mean ± SEM unless otherwise noted.

**Results**

**PKCε is activated in response to IR and is required for IR-induced apoptosis in salivary gland acinar cells**

We have previously shown that PKCε/− mice are resistant to IR-induced damage to the salivary gland and thymus and have a delay in mammary gland involution, a process driven by apoptosis (6, 11). Our studies demonstrate that phosphorylation of PKCε at Y64 and Y155 by c-Src and c-Abl, respectively, drives nuclear translocation and is necessary and sufficient for the proapoptotic function of PKCε (5, 7, 8). To determine whether PKCε is activated in response to IR, we examined phosphorylation of PKCε at T505 in the activation loop of the kinase, and at Y64 and Y155 in the regulatory domain. Phosphorylation of PKCε at T505 is seen within 1 hour after IR, while phosphorylation at Y64 and Y155 is detectable by 2 hours post-IR and continues to increase until at least 6 hours (Fig. 1A). To determine whether PKCε is required for IR-induced apoptosis, we assayed caspase activation in cells depleted of PKCε with specific shRNAs (shδ1 and shδ3) or a scrambled shRNA (shScr). Expression of shδ3 reduced apoptosis by up to 50% relative to cells transfected with a scrambled shRNA control, and a similar trend was seen with shδ1 (Fig. 1B).

**TKIs block activation of PKCε and suppress apoptosis**

We have previously shown that both dasatinib and imatinib can block phosphorylation of PKCε at Y64 and Y155, nuclear translocation of PKCε, and apoptosis in response to DNA-damaging agents (5). Here, we show that pretreatment of ParC5 cells with dasatinib, imatinib, or bosutinib, all of which target c-Src and/or c-Abl, inhibits IR-induced apoptosis (Fig. 1C–E). IR-induced apoptosis was reduced 40% to 60% by all TKIs, with bosutinib and imatinib being more potent than dasatinib. Likewise, activation of c-Abl and c-Src (SKF, Src family kinases) and phosphorylation of PKCε at Y155 and Y64 were also suppressed by pretreatment with TKIs (Fig. 1F–H). Interestingly, dasatinib and imatinib were slightly more potent inhibitors of Y155 phosphorylation than Y64 phosphorylation, consistent with their more potent inhibition of c-Abl than c-Src in ParC5 cells (Fig. 1F and G).

**TKIs protect salivary gland function in mice treated with head and neck IR**

To explore radioprotection by TKIs in vivo, we focused on dasatinib, a broad-spectrum TKI, and imatinib, which preferentially inhibits c-Abl tyrosine kinase. The schematic in Fig. 2A depicts the three dosing regimens used for the experiments in Fig. 2. To determine whether dasatinib (Fig. 2B) or imatinib (Fig. 2C) can protect salivary gland function, mice were dosed 1 hour before and 3 hours following IR delivery ("pre/post"), and production of whole saliva was assayed after 60 days. Although whole saliva includes secretions from the three major salivary glands (submandibular, parotid, and sublingual), the majority is produced by the submandibular glands (SMG). In mice that received IR plus vehicle, the saliva production was significantly reduced at 60 days post-IR, reaching only 30% of the vehicle-treated mice (Fig. 2B). Dasatinib administration partially prevented the IR-mediated decrease in saliva production, and these mice produced 65% of the vehicle-treated controls (Fig. 2B). Salivary gland function was protected to even a greater extent in mice treated with imatinib in conjunction with IR (Fig. 2C); these mice retained >90% of their salivary function when treated with imatinib plus IR compared with IR alone.

We next asked whether treatment with a single dose of dasatinib or imatinib prior to IR is sufficient for radioprotection (see Fig. 2A "pre"), and whether delivery of dasatinib or imatinib after IR affords radioprotection (see Fig. 2A "post/post"). In the experiment shown in Fig. 2D, saliva production after IR treatment was reduced by 70%. Remarkably, a single dose of dasatinib given prior to IR (pre), or two doses of dasatinib given post-IR (post/post), offered similar protection as dosing mice pre/post-IR (Fig. 2D). All three treatment protocols with dasatinib resulted in nearly 100% protection of salivary gland function compared with vehicle-only–treated mice (Fig. 2D). Similar results were seen in mice treated with imatinib (Fig. 2E). Salivary gland function was >80% of the vehicle control with pre/post or post/post-delivery of imatinib, while one dose prior to IR (pre) resulted in salivary production 63% of the vehicle control (Fig. 2E).

In patients, decreased salivary gland function is seen in the first weeks after IR and is presumably due apoptosis of IR-damaged cells. In contrast, the inability to regenerate new salivary acinar cells presumably contributes to the permanent salivary gland hypofunction seen in many patients (2). Our studies demonstrate that dasatinib and imatinib can provide robust protection of salivary gland function for at least 60 days after delivery of IR. To address whether radioprotection persists beyond this time, we compared the effect of IR on saliva production in mice that received the pre/post treatment alone to mice that received the pre/post regimen and then continued to receive dasatinib twice a week for up to 5 months following a single dose of IR (see Fig. 3A for scheme). As seen in Fig. 3B, salivary function in mice that received IR alone was 50% of vehicle-treated mice at 30 days, and decreased to 20% at 150 days. In comparison, in mice dosed only before and after IR (pre/post), saliva production at 30 and 60 days was 70% of vehicle-treated mice, but declined thereafter (Fig. 3B). Saliva production in this group was not significantly different from IR alone at 90 and 120 days. In contrast, mice that received continual dosing with dasatinib maintained a significantly higher level of salivary function out to 5 months. In this group, saliva production was 74% of vehicle-treated controls at 90 days, 69% at 120 days, and 82% at 150 days after IR (Fig. 3B).

Protection of salivary gland function in mice treated with dasatinib plus IR is suggestive of salivary gland tissue preservation, and/or increased gland regeneration. To ask whether increased saliva production correlates with an increase in salivary acinar cell number, the experiment shown in Fig. 3B was terminated after 150 days, and the salivary glands were analyzed histologically. Staining with H&E revealed a decrease in salivary gland acinar cells in the SMGs of mice treated with IR alone; however, this appeared to be largely reversed in mice that received continuous dosing with dasatinib (Fig. 3C). To quantify the salivary-producing acinar cells, tissue sections from the entire salivary gland were stained by IHC for expression of aquaporin 5 (AQP5), a specific acinar cell marker, and the relative expression of AQP5 across different treatment groups was quantified using Aperio Image Analysis software. In IR-
treated mice, the SMG acinar cell population was reduced by 60% compared with the SMGs of unirradiated mice (Fig. 3C and D). This correlates with the 80% decrease in saliva production we reported in the IR alone–treated mice (Fig. 3B). Dasatinib treatment pre/post-IR was not sufficient to prevent the decrease in acinar cells, consistent with our finding that in this treatment group, saliva production was not different from the IR alone group at 150 days (Fig. 3B). However, in IR-treated mice continuously treated with dasatinib, the amount of AQPN5 staining in the SMGs was 93% of that observed in vehicle-treated mice, and consistent with protection of salivary gland function in this treatment group (Fig. 3B).
Inhibition of PKCδ or treatment with TKIs does not promote survival of HNSCC cells

To be of benefit to patients, strategies to protect radiosensitive nontumor tissues must not impact tumor eradication. To address this, we examined clonogenic survival and activation of cell survival pathways in Cal27, FaDu, and UMSCC19 HNSCC cell lines stably depleted of PKCδ, and in the context of TKI pretreatment. Depletion of PKCδ had no impact on the clonogenic survival of any of the HNSCC cell lines analyzed (Fig. 4A–C). Similarly, there was no change in clonogenic survival following IR of Cal27, FaDu, or UMSCC19 cells treated with dasatinib, imatinib, or bosutinib (Fig. 4D–F). To confirm that TKI treatment does not affect prosurvival signaling, Cal27, FaDu, and UMSCC19 cells were treated with dasatinib, IR, or dasatinib plus IR. As seen in Fig. 4G–I, although IR resulted in a modest increase in pAkt and/or pERK in Cal27 and FaDu cells, the addition of dasatinib had no effect, or reduced the level of activation.

TKIs do not enhance tumor growth when combined with fractionated IR

In patients, IR is typically delivered in multiple fractions over 4 to 6 weeks. To assess the impact of TKIs on salivary gland function under similar conditions, we used a fractionated model where mice received 4 Gy IR to the head and neck on each of 5 consecutive days. Mice were dosed with imatinib before and after (pre/post) each fraction of IR. As shown in Fig. 5A, in mice treated with IR alone, saliva production was reduced by 60% compared with the vehicle control group. However, in mice treated with IR plus imatinib, salivary gland function at 60 days after IR was not significantly different from the vehicle treatment alone, or imatinib only control groups, indicating that imatinib can provide protection of salivary gland function in a fractionated IR model.

We next asked whether TKIs impact the growth of HNSCC tumor xenografts when delivered in combination with fractionated IR. FaDu cells were grown as flank xenograft tumors and mice were given 6 Gy of IR at the tumor site for 5 consecutive days. Before and after each IR, treatment mice were dosed with either imatinib or dasatinib (pre/post dosing). Fractionated IR alone resulted in the attenuated growth of FaDu-derived xenograft tumors (Fig. 5B), and the addition of either dasatinib or imatinib had no effect on tumor growth. Interestingly, in mice treated with either dasatinib or imatinib alone, there was a trend toward reduced tumor growth compared with their respective controls. Together, these data support the conclusion that TKIs do not enhance tumor growth when combined with fractionated IR.
demonstrate that PKCδ inhibition is effective in protecting salivary gland function without sacrificing the cytotoxic effect of IR on HNC and support evaluation of TKIs for radioprotection of the salivary gland in patients receiving IR for HNC.

**Discussion**

Nearly 60% of cancer patients will require IR at some point during their treatment, many of whom will suffer significant, and sometimes permanent, side effects due to IR damage to healthy nontumor tissues in the radiation field (2). Acutely radiosensitive tissues that present significant clinical challenges include the hematopoietic system, gastrointestinal mucosa, and tissues in the oral cavity (1). Unfortunately, there is little to offer these patients, underscoring the need for new therapeutic strategies that provide selective protection of radiosensitive normal tissues without impacting tumor cell death. We have previously shown that PKCδ is required for IR-induced apoptosis in the salivary gland and that blocking activation of PKCδ with TKIs suppresses apoptosis (5).

In this study, we show that dasatinib and imatinib provide profound and durable protection of salivary gland function in vivo when delivered in conjunction with a single or fractionated doses of IR.

Although most damage to nontumor tissues in the oral cavity resolves in the months following IR, damage to the salivary gland, and the resulting xerostomia ("dry mouth syndrome") can be permanent, particularly for those with locally advanced HNC (15, 16). Considerable effort has been put into restoring the function of IR-damaged glands by gene transfer of AQPN5 into residual ductal cells, or regeneration of salivary gland tissue for reimplantation into IR-damaged glands (17, 18). Both of these approaches show remarkable promise for patients who suffer from severe loss of salivary gland function. However, the development of radioprotection agents that prevent IR-induced salivary gland damage is still a pressing need, and the availability of such agents would largely alleviate the need to repair or regenerate damage tissues. Attempts to protect salivary gland function have focused on use of free radical scavengers, such as amifostine (4) to reduce cell injury, and growth factors such as FGR, IGF, and KGF to stimulate gland regeneration (19–22). Our approach to reducing IR toxicity to the salivary gland is based on inhibiting the activation of PKCδ,
a proapoptotic protein kinase required for DNA damage–induced apoptosis in vitro and in vivo (23).

Tissue damage following IR is thought to be driven primarily by irreversible cell damage to replicating cells resulting in cell-cycle arrest and apoptosis. Although radiosensitive tissues typically have a high rate of cell turnover, the salivary glands are composed of highly differentiated cells that are thought to be primarily postmitotic. In spite of this, widespread apoptosis is seen in the salivary glands of mice treated with 25 Gy of IR, especially within the first 24 hours (5). We have previously shown that activation of PKCδ by DNA-damaging agents requires tyrosine phosphorylation by c-Abl and c-Src and that TKIs of c-Abl and c-Src can block activation of PKCδ and DNA damage–induced apoptosis in vivo (5). In our current studies, we show that three different TKIs can suppress activation of PKCδ and apoptosis induced by IR in vitro and demonstrate the efficacy of TKIs in radioprotection in vivo. A single dose of dasatinib or imatinib given prior to IR was sufficient to protect salivary gland function at least 60 days (Fig. 2). Notably, delivery of TKIs at 3 and 6 hours post-IR also protected salivary gland function, suggesting that TKIs may be useful in mitigating IR damage (Fig. 2D and E). All TKIs tested are relatively broad spectrum, consistent with their ability to inhibit IR-induced activation of both c-Abl and c-Src, and to suppress phosphorylation of both Y155 and Y64 on PKCδ (Fig. 1C). Previous studies have also focused on inhibition of apoptosis as a strategy for radioprotection, particularly through inhibition of p53 and p53 target genes (24, 25). Notably, the proapoptotic function of PKCδ has been linked to the downstream activation of p53, and its transcriptional targets (p21 and PUMA), suggesting a mechanism through which suppression of PKCδ may reduce the apoptotic response (26, 27). A report by Barckhausen and colleagues also demonstrates that inhibition of apoptosis can promote DNA repair (28), consistent with a reported role for PKCδ in regulation of DNA repair (23).
Loss of salivary function following IR occurs more rapidly than can be accounted for by cell turnover alone, and models of "early" and "late" effects of IR have been proposed to explain this conundrum (29). Early effects of IR are seen within the first few weeks and likely result from direct damage to acinar cells resulting in impairment of secretory ability, and the subsequent loss of cells through apoptosis. In contrast, late effects presumably reflect an inability to regenerate new secretory cells due to loss of an epithelial progenitor population. Other "late" effects in the gland, including inflammation and fibrosis, may also impact the regenerative capacity of the gland. In our studies, salivary function is decreased by >50% at 30 days after IR and continues to decrease for at least 50 days, at which time only 20% of the functional capacity remains (Fig. 3B). This is consistent with a decrease in AQPN5 expression, which marks the secretory acinar cells, within the SMG (Fig. 3D). Although pre/post-dosing with dasatinib protects function at 30 and 60 days, this effect is largely lost thereafter. On the basis of our studies demonstrating a critical role for PKCδ in apoptosis in the salivary gland in vivo, and studies in this article that demonstrate that TKIs suppress IR-induced apoptosis (Fig. 1B), we propose that suppression of acinar cell apoptosis contributes to the radioprotection observed in the first 30 to 60 days after IR by helping to maintain the secretory capacity of the gland. However, as salivary function continues to decline beyond 60 days, many of these cells must be eventually lost and not replaced.

In contrast to the pre/post-delivery of dasatinib, we show that continuous dosing of dasatinib in combination with IR results in sustained radioprotection and maintenance of SMG acinar cell number (Fig. 3D). This suggests that TKIs may impact the regenerative capacity of the salivary gland. The simplest explanation is that TKIs protect a stem/progenitor cell pool that can be used to replace cells lost acutely through apoptosis. In this regard, reports using H1 and BrdUrd labeling demonstrate an acinar cell subpopulation within the SMG capable of undergoing proliferation (30), and a number of groups have identified and cultured stem cell populations from rodent and human salivary glands (31). Notably, PKCδ has been shown to be important in the proliferation of stem cells from breast, pancreatic, prostate, and melanoma tumors (32). More recently, Aure and colleagues described the ability of labeled acinar cells to undergo self-duplication within the parotid and submandibular glands (33). TKIs may also contribute to regeneration by suppressing IR-induced changes in the microenvironment. Studies in the skin, lung, and kidneys show that IR can induce a chronic oxidative environment resulting in changes in the expression of proinflammatory cytokines and a subsequent increase in apoptosis (34–37). Dasatinib is capable of interfering with the inflammatory response through inhibition of TNFα, TGFβ, and CXCR4 (38–40).

Our studies demonstrate the potential for TKIs to be used in vivo as a novel therapeutic approach for radioprotection of salivary gland function without accelerating tumor growth. In fact, our data suggest a trend toward suppression of tumor growth in mice treated with dasatinib or imatinib alone (Fig. 5B). This is consistent with clinical data where dasatinib given as a single agent failed to inhibit tumor growth, but also did not enhance tumor growth (41, 42). The wide use of radiotherapy for various cancer patients suggests that TKIs may have broad implications in preventing harmful IR-related side effects. A recent report by Aigueperse and colleagues shows that the administration of dasatinib and the inhibition of c-Src protects against intestinal IR injury (43). In addition, our results may have implications for side effects that result from chemotherapy, as a recent report by Pabla and colleagues demonstrates that PKCδ inhibition provides protection against cisplatin-induced nephrotoxicity while simultaneously improving its cytotoxicity in tumor cells (44). These previous studies along with our current findings suggest that inhibition of PKCδ may be a promising therapeutic strategy for the protection of non-tumor tissues in patients undergoing cancer therapy.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: S.M. Wie, M.E. Reyland
Development of methodology: S.M. Wie, E. Wellberg, M.E. Reyland
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.M. Wie, E. Wellberg, M.E. Reyland
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.M. Wie, S.D. Karam, M.E. Reyland
Writing, review, and/or revision of the manuscript: S.M. Wie, E. Wellberg, S.D. Karam, M.E. Reyland

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