Inhibition of Fibroblast Growth Factor Receptor Attenuates UVB-Induced Skin Carcinogenesis

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Altered fibroblast GF receptor (FGFR) signaling has been shown to play a role in a number of cancers. However, the role of FGFR signaling in the development and progression of UVB-induced cutaneous squamous cell carcinoma remains unclear. In this study, the effect of UVB radiation on FGFR activation and its downstream signaling in mouse skin epidermis was examined. In addition, the impact of FGFR inhibition on UVB-induced signaling and skin carcinogenesis was also investigated. Exposure of mouse dorsal skin to UVB significantly increased the phosphorylation of FGFRs in the epidermis as well as the activation of downstream signaling pathways, including protein kinase B/mTOR, signal transducers and activators of transcription, and MAPK. Topical application of the pan-FGFR inhibitor AZD4547 to mouse skin before exposure to UVB significantly inhibited FGFR phosphorylation as well as mTORC1, signal transducer and activator of transcription 3, and MAPK activation (i.e., phosphorylation). Moreover, AZD4547 pretreatment significantly inhibited UVB-induced epidermal hyperplasia and hyperproliferation and reduced the infiltration of mast cells and macrophages into the dermis. AZD4547 treatment also significantly inhibited mRNA expression of inflammatory genes in the epidermis. Finally, mice treated topically with AZD4547 before UVB exposure showed decreased cutaneous squamous cell carcinoma incidence and increased survival rate. Collectively, the current data support the hypothesis that inhibition of FGFR in the epidermis may provide a new strategy to prevent and/or treat UVB-induced cutaneous squamous cell carcinoma.

INTRODUCTION

Cutaneous squamous cell carcinoma (cSCC) is a keratinocyte (KC)-derived invasive and metastatic tumor of the skin (Farshchian et al., 2015). cSCC comprises 20% of the 1.3 million nonmelanoma skin cancers that arise each year in the United States. The great majority of cases are effectively managed with surgery and other local therapy. However, management of locally advanced or metastatic disease still remains an ongoing clinical challenge. Furthermore, organ transplant recipients are 65–250 times at a greater risk for cSCC than the general population and have over five times the rate of cSCC compared with the rates of all cancers combined in the general public. Cemiplimab, an immune checkpoint blockade PD-1 inhibitor, is the first and only Food and Drug Administration drug approved just last year for the treatment of aggressive cSCC. However, immune checkpoints play a role in preventing a patient’s immune system from rejecting transplanted organs, and further research is needed to determine the overall safety of these agents in this setting. cSCC of the head and neck is even more challenging because reconstruction of facial defects after excision can be complex and have a long-term impact on the patient’s QOL. More importantly, many patients exhibit condemned skin with multiple cSCC of the face, leading to facial disfigurement (Parikh et al., 2014). An improved understanding of the molecular mechanisms underlying skin carcinogenesis is critical for developing novel strategies for both the prevention and treatment of this disease.

Fibroblast GF receptors (FGFRs) are transmembrane receptor Y kinases of the Ig superfamily. In humans, the FGFR family consists of four genes encoding closely related transmembrane receptor Y kinases (Johnson and Williams, 1993). The binding of fibroblast GF ligands to the FGFRs induces receptor dimerization, leading to conformational changes that enable transphosphorylation of Ys in the intracellular...
domain, including the kinase domain and the C-terminus. Fibroblast GFs and their receptors convey multiple biological activities, including proliferation, differentiation, and motility (Grose and Dickson, 2005). Phosphorylation of FGFR triggers multiple intracellular signaling cascades, including but not limited to RAS-RAF/MAPK-extracellular signal-regulated kinase (ERK) kinase, phosphoinositide 3-kinase/protein kinase B (Akt), signal transducer and activator of transcription 3 (STAT3), and NF-kB pathways (Ahmad et al., 2012). Interestingly, the signaling mechanism is further complicated with alternating splicing of the receptors and ligand redundancy (Ornitz and Itoh, 2015). FGFR transcript undergoes alternative splicing resulting in IIib and IIc isoforms. Elevated FGFR levels have been found in a number of cancers, including prostate cancer, breast cancer, lung cancer, brain cancer, gastric cancer, sarcoma, and head and neck cancer (Chesi et al., 1997; Freier et al., 2007; Marek et al., 2009). Recent studies show that amplification, abnormal activation, or SNPs in the FGFR2 gene are important in cancer progression (Byron et al., 2008; Easton et al., 2007; Hunter et al., 2007). Furthermore, a dramatic increase in FGFR2 expression was noted selectively within malignant epithelial cells, suggesting FGFR2 as a potential unexplored therapeutic target in cSCC (Ramsey et al., 2013).

Chronic exposure to UVR is the primary cause of melanoma and nonmelanoma skin cancers. Development of UVB-induced skin carcinoma is a multistep and complex process. In humans, the most damaging effects are caused by UVB. A total of 99% of UVB (290–320 nm) is absorbed to a depth of 40 μM in the epidermis and is responsible for erythema (sunburn) and ultimately development of actinic keratosis, basal cell carcinoma, cSCC, and melanomas. Similar to chemical carcinogenesis, UVB light acts as a tumor initiator by producing irreversible mutagenic damage to the epidermal cells through unrepaired photoproducts, photohydrates, and oxidative damage (Abel et al., 2009). Furthermore, UVB-induced tumor initiation is associated with sequence variation at critical target genes, such as p53, H-RAS, and N-RAS. Subsequent exposure to UVB can lead to tumor promotion by inducing morphological and biochemical changes that cause expansion of the initiated cell population and changes in cell behavior. These tumor promotion events include but are not limited to dermal inflammation, epidermal hyperplasia, increased ornithine decarboxylase expression and activity, and altered cell cycling. By increasing epidermal hyperplasia, UVR can also increase the clonal expansion of the initiated cell, leading to malignant conversion and metastasis (Macias et al., 2013).

In our recently published study, oral administration of a pan-FGFR inhibitor (AZD4547) inhibited UVB-induced acute skin changes in adult mice, suggesting a causative role for FGFR in UVB-induced skin carcinogenesis. However, the efficacy and mechanism for topical AZD4547 application are not yet ascertained. Accordingly, we sought to investigate the role of topical application of AZD4547 on the classical FGFR signaling and other oncogenic pathways in epidermal KCs. Furthermore, the efficacy of AZD4547 in inhibiting UVB-induced skin changes such as epidermal hyperproliferation, hyperplasia, and cell signaling pathways was determined. Finally, the role of AZD4547 in modulating UVB-induced skin carcinogenesis was investigated.

RESULTS
UVB-induced activation of epidermal FGFRs
To fully understand the effects of UVB exposure on FGFR signaling pathways, changes in total protein levels and phosphorylation status of FGFRs and FRS2α were evaluated. After exposure of mouse skin to UVB given three times per week for 2 weeks, the epidermis was collected at different time points (from 0 to 24 hours), and epidermal protein lysates were prepared. As shown in Figure 1a, p53 used as a DNA damage marker and as a positive control for UVB exposure was upregulated in the epidermis of UVB-exposed mice in comparison with that of the sham control group. Western blot analyses showed that FGFR1, 2, and 3 proteins were expressed in the epidermis and that the levels remained unchanged after exposure to UVB (Figure 1a and Supplementary Figure S1a). In contrast, FGFR4 protein was not detected in mouse epidermis (data not shown). The phosphorylation levels of FGFR1/2 (Y653/654) and FRS2α (Y196 & Y436) were significantly ($P \leq 0.05$) elevated as early as 2 hours after the last UVB treatment and remained elevated up to 24 hours compared with the levels in the sham-treated control group (again, see Figure 1a and Supplementary Figure S1a). We next sought to investigate the effect of UVB on FGFR ligands. As shown in Figure 1b and Supplementary Figure S1b, exposure to UVB led to the upregulation of FGFR ligand protein levels in the epidermis. After the last treatment of UVB, protein levels of FGF2, 7, and 8 were significantly elevated at 2 hours and remained elevated for at least 24 hours. These data are consistent with the activation of FGFRs as shown in Figure 1a, especially FGFR2. Supplementary Figure S2 also shows that FGF10 (KGF-2), an FGFR2b ligand (Eswarakumar et al., 2005; Mohammadi et al., 2005), was also upregulated in the epidermis after exposure to UVB.

Activation of downstream epidermal signaling pathways after UVB exposure
Exposure of mouse skin to UVB activates a number of oncogenic signaling pathways (Rho et al., 2011). To further explore the role of FGFRs in UVB-induced cell signaling, we characterized a number of oncogenic signaling pathways at different time points in the epidermis after the last treatment with UVB using multiple treatment protocols. As shown in Figure 2a and Supplementary Figure S3a, various components of the Akt/mTORC1 pathway showed significant ($P \leq 0.05$) increases in phosphorylation: Akt (Th308 and S473), PRAS40 (Th246), r-S6 (S235/236), 4EBP1 (S65), and p70S6K (Th389). Analysis of STAT signaling revealed increased activation (phosphorylation) of STAT1 (Y701), STAT3 (Y705), and STAT5 (Y694) compared with that in the control group. The activation (phosphorylation) of STAT1 (Y701), STAT3 (Y705), and STAT5 (Y694) compared with that in the control group. The levels of phosphorylated Jak2 protein levels did not change after UVB exposure, suggesting that the activation of STATS observed does not involve this kinase (Figure 2b and Supplementary Figure S3b).

MAPK signaling was also upregulated in the epidermis of skin exposed to UVB protocol (Figure 2c and Supplementary Figure S3c). Phosphorylated MAPK/ERK kinase 1/2 was significantly elevated at all time points up to 24 hours. Similar results were observed for the protein levels of both dual-specificity phosphatase 6 (DUSP6) and c-Jun. In contrast,
phosphorylated ERK1/2 levels remained unchanged at the early time points and then slightly decreased at later time points.

**Effect of AZD4547 pretreatment on FGFR activation and downstream signaling pathways induced by UVB**

Using the same multiple exposure protocol, the effect of topical application of AZD4547 on UVB-induced signaling pathways was evaluated. The data in Figures 1 and 2 indicated that the maximal UVB effect for activation of FGFRs and most signaling pathways was between 2 and 4 hours after the last UVB exposure. Hence, we pretreated the mice topically with AZD4547 (200 and 400 μg dissolved in 200 μl of acetone vehicle) 1 hour before UVB exposure, and the samples were collected 4 hours after the last UVB treatment. AZD4547, at a topical dose of 400 μg per mouse, significantly decreased the phosphorylation of FGFR1/2 (Y653/654), 4EBP1 (S65), p70S6K (T389), and r-S6 (S2235/236) (Figure 3a and b). Total protein levels of FGFR2 remained unchanged. Further western blot analyses also revealed that topical application of AZD4547 at the 400 μg per mouse dose remarkably reduced the phosphorylation of STAT3 (Y705 and S473) and had no effect on total STAT3 levels (Figure 4a and b). These data show that activation of FGFRs and downstream signaling pathways after exposure to UVB exposure can be modulated using AZD4547.

For the experiments in Figures 3 and 4, p53 levels in the epidermis from UVB-treated mice were again examined. Similar to the data in Figure 1, p53 protein levels were elevated as expected; however, topical application of AZD4547 (400 μg) significantly reduced UVB-induced p53 protein levels in comparison with that in the acetone control group (Figure 3a). This observation suggested a possible photoprotective effect of topically applied AZD4547. To explore this possibility, we stained the skin sections from UVB-treated mice that received AZD4547 pretreatment and evaluated cyclobutane pyrimidine dimer formation by immunohistochemistry. As shown in Supplementary Figure S4, there were no significant differences in cyclobutane pyrimidine dimer-positive cells comparing the acetone vehicle group with the AZD454-treated groups at either dose. These results suggest that topical AZD4547 did not have a significant photoprotective effect. These data indicate that the decrease in p53 observed after pretreatment with AZD4547 is due to inhibition of FGFRs and not due to a photoprotective effect.
The effect of topical AZD4547 on epidermal VEGFR2 phosphorylation was also evaluated on the basis of reports of activity against this receptor in other cells and tissues (Gavine et al., 2012; Le et al., 2017). As shown in Supplementary Figure S5, phosphorylated VEGFR2(Y1175) was elevated after exposure to UVB, and topical pretreatment with AZD4547 (400 mg) significantly decreased the phosphorylation levels of this receptor.

Effect of AZD4547 on epidermal proliferation and skin inflammation

We evaluated the effect of UVB with or without AZD4547 pretreatment on epidermal proliferation and skin inflammation after exposure to UVB. Immunohistochemistry analyses were performed on mouse skin sections collected 4 hours after the last treatment of UVB. BrdU was used as a proliferation marker (Figure 5a), and quantitation of immunohistochemistry images showed that pretreatment with AZD4547 (both 200 and 400 μg) significantly reduced epidermal hyperproliferation and hyperplasia (Figure 5b and c). Immunohistochemistry analyses also revealed the infiltration of mast cells and macrophages (again, see Figure 5a) in the dermis in response to UVB exposure. Pretreatment with AZD4547 (both 200 and 400 μg) significantly decreased the infiltration of mast cells and
macrophages into the dermis (Figure 5d and e). The effect of AZD4547 on skin inflammatory cell infiltrate led us to examine its effect on the expression of a number of inflammatory cytokines, chemokines, and other inflammation-associated genes. RT-qPCR analysis showed elevated mRNA expression of Tnfa, Ccl2, Il1a, Cxcl9/10/11, Nkbi, Pdli, and Cox2 in epidermal RNA samples after treatment with UVB compared with the expression in the sham-treated control (Supplementary Figure S6). Topical pretreatment with AZD4547 (400 µg) before UVB exposure reduced the level of all mRNAs examined, consistent with an overall decrease in inflammation observed. Topical treatment with AZD4547 in the absence of UVB treatment led to a modest ~1.4-fold increase in Pdli mRNA and ~2-fold increase in Ccl2 mRNA. Both of these increases were statistically significant.
Effect of topical AZD4547 treatment on UVB skin carcinogenesis

Given the significant inhibitory effects of topical AZD4547 on UVB-induced epidermal FGFR signaling and epidermal proliferation and inflammation, the effect of this compound (400 µg per mouse given 1 hour before each UVB treatment) on UVB skin carcinogenesis was evaluated. Briefly, mice were topically pretreated with AZD4547. One hour after treatment, mice were exposed to 1,200 J/m² of UVB three times per week. The dose was increased gradually by increments of 25% weekly until a maximum dose of 4,500 J/m² of UVB was reached. This protocol has been described by us previously (Eguiarte-Solomon et al., 2021; Kim et al., 2009a). As shown in Figure 6, topical AZD4547 treatment significantly increased tumor-free survival (P = 0.002) (Figure 6a) and reduced the number of tumors (squamous cell carcinoma...
per mouse to 1.0 ± 0.21 compared with 2.2 ± 0.44 in the control group (P = 0.0387) (Figure 6b). All tumors were verified as SCC by histopathologic diagnoses and positive staining for keratin and negative staining for vimentin (Figure 6c)

DISCUSSION
Numerous clinical trials evaluating the safety and efficacy of FGFR inhibitors such as pemigatinib and AZD4547 to treat solid cancers are currently underway. Some of the notable studies include clinical trials evaluating the effectiveness and safety of pemigatinib in participants with previously treated locally advanced and metastatic or surgically unresectable solid tumor malignancies harboring activating FGFR mutations or translocations (NCT03822117). Recently, the Food and Drug Administration granted accelerated approval to pemigatinib for cholangiocarcinoma with an FGFR2 rearrangement or fusion (Abou-Alfa et al., 2020). In addition, a phase II trial is currently evaluating the efficacy of pemigatinib in patients with unresectable and metastatic colorectal cancer with mutations (alterations) in an FGFR gene (NCT04096417). Similarly, AZD4547, a pan-FGFR inhibitor, exhibited promising results in the National Cancer Institute–Molecular Analysis for Therapy Choice trial (EAY131) subprotocol W, suggesting efficacy in breast (33.3%), urothelial (12.5%), and cervical (10.4%) cancers with FGFR activating mutations and fusions (Chae et al., 2020). Furthermore, evidence exists for the inhibitory role of AZD4547 in pediatric solid tumors, breast cancer, non-small cell lung cancer, endometritis, and head and neck...
The FGFRs are involved in the regulation of normal skin development and have also been implicated as having either a causative or suppressive role in skin cancer (Czyz, 2019). The efficacy of AZD4547 in the prevention of UVB-induced skin carcinogenesis is not yet elucidated. Unfortunately, oral administration of FGFR inhibitors may be associated with severe side effects that can be limited through topical administration in tumors such as cSCC (Saka et al., 2017). To our knowledge, the significance of FGFR receptors and the use of AZD4547 in UVB-induced acute effects in the epidermis and in UVB-induced skin carcinogenesis has not been reported.

As shown in Figure 1, FGFR1, 2, and 3 were detected in epidermal protein lysates from control and UVB-treated mouse skin, whereas FGFR4 was not detectably present. Exposure of mouse skin to UVB led to increased

Figure 6. Topical AZD4547 inhibits UVB-induced SCC incidence and multiplicity. Female FVB/N mice were treated topically with either AZD4547 (400 µg/100 µl) or acetone (100 µl), followed by exposure to increasing doses of UVB (1,200 J/m² – 4,500 J/m²). (a) Data represents Kaplan–Meier tumor-free survival curves for n ≥ 9 mice per group. Data were analyzed using the Log-rank (Mantel–Cox) test for statistical significance (Prism 5, GraphPad Software, San Diego, CA). P = 0.0002. (b) The average number of skin tumors per mouse (mean ± SEM, n ≥ 9 per group, *P = 0.0387, Mann–Whitney U test). (c) Representative SCCs immunostained for (i) H&E, (ii) pan-keratin (VentanaMed 760-2595), and (iii) vimentin (VentanaMed 790-2917), followed by DAB as the chromogen confirming the squamous cell origin of the tumors. Bar = 100 µm. DAB, 3,3'-diaminobenzidine; SCC, squamous cell carcinoma.
phosphorylation (activation) of FGFRs. The increased phosphorylation of epidermal FRS2α (at both Y196 and Y436) provided further evidence of epidermal FGFR activation after exposure to UVB. The lack of individual FGFR receptor–specific phosphorylation antibodies precluded further determination of which FGFRs were activated. However, UVB exposure was also found to upregulate FGFR ligands in the epidermis, including FGF7 (also known as KC GF or KGF1) and FGF10 (KGF2) that specifically binds to FGFR2 (Ilb) (Eswarakumar et al., 2005; Mohammadi et al., 2005), and is consistent with activation of epidermal FGFR2 after exposure of mouse skin to UVB (Figure 1 and Supplementary Figure S2). In addition, FGF2 and FGF8 were also upregulated in the epidermis of UVB-treated skin. Upregulation of these latter fibroblast GFs is consistent with the possibility that other FGFRs such as FGFR1 and FGFR3 were also activated in the epidermis after exposure to UVB. Topical application of AZD4547 (400 μg per mouse) significantly reduced the phosphorylation of FGFRs by ~50% (Figure 3). This effect of AZD4547 was not due to a sunscreen effect because we did not observe a statistically significant reduction in UVB-induced cyclobutane pyrimidine dimers (Supplementary Figure S4B) after topical application of this compound.

As shown in Figures 2–4, topical application of AZD4547 inhibited a variety of oncogenic signaling pathways such as Akt–mTORC1, MAPK, and STAT3 known to play an important role in UVB-induced skin carcinogenesis (Rho et al., 2011). These data are consistent with our previously published results using the oral route for administration of AZD4547 (Khandelwal et al., 2016). These data provide evidence that activation of FGFRs after exposure of mouse skin to UVB may be responsible, in part, for activation of the Akt–mTORC1, MAPK, and STAT pathways during UVB skin carcinogenesis. Studies published from our laboratory and others have documented the role of STATs in various biological activities, including cell proliferation, migration, and survival (Chan et al., 2004; Kim et al., 2009b). Furthermore, studies using KC-specific STAT3–deficient mice have revealed that STAT3 plays an essential role in skin homeostasis, including KC migration, wound healing, and hair follicle growth. The use of both constitutive and inducible KC–specific STAT3-deficient mouse models has shown that STAT3 is required for both the initiation and promotion stages of multistage skin carcinogenesis as well as for UVB skin carcinogenesis (Kim et al., 2009b). STAT5, including STAT3, are known to be activated downstream of FGFRs (Song et al., 2021). We have now shown that topical treatment with AZD4547 inhibited UVB-induced phosphorylation of STAT3.

Previous studies from our laboratories have shown that STAT3 plays a critical role in regulating KC proliferation and survival after exposure to UVB (Kim et al., 2009a; Sano et al., 2008). Previous studies, including work from our laboratories, have also shown that UVB activates the oncogenic RAS–MAPK and phosphoinositide 3-kinase–Akt/mTORC1 signaling pathways in epidermal KCs (Carr et al., 2012). FGFR activation leads to the phosphorylation of adapter proteins required for the intracellular oncogenic signaling pathways. Activated (phosphorylated) FRS2α binds the membrane-anchored adaptor protein, GRB2, and the tyrosine phosphatase, SHP2. GRB2 further activates the RAS–MAPK pathway by recruiting SOS and the phosphoinositide 3-kinase–Akt pathway by recruiting GAB1 to the signaling complex (Kouhara et al., 1997; Timsah et al., 2016). As shown in Figures 3 and 4, AZD4547 significantly attenuated UVB-induced mTORC1 and MAPK activation in the epidermis. Both of these pathways are also known to contribute to epidermal proliferation during UVB-induced skin carcinogenesis (Anwar et al., 2020; Carr et al., 2012). 

DUSP6 and EGR1 are transcript biomarkers of the FGFR signaling pathway, modulated by AZD4547 (Delpuech et al., 2016). DUSP6, also known as MKP3, inhibits ERK1/2 and negatively regulates FGFR signaling (Li et al., 2007). This phosphatase was significantly upregulated after UVB exposure, and topical AZD4547 treatment significantly inhibited its upregulation. This effect on DUSP6 further led to the downregulation of EGR1. Among FGFR-regulated genes, EGR1 in particular is shown to be altered specifically by FGFR2 signaling (Delpuech et al., 2016). Thus, inhibition of multiple downstream FGFR signaling pathways likely contributed to the inhibitory effect of topical AZD4547 on KC proliferation induced after UVB exposure (Figure 5).

Recent studies have also highlighted the role of FGFRs in inflammation (Wang et al., 2020). For example, inhibition of FGFR signaling inhibited inflammation after acute hepatic injury by suppressing the release of proinflammatory cytokines. Besides inflammation, FGF/FGFR signaling is also associated with angiogenesis at multiple levels (Ichikawa et al., 2020; Li et al., 2002). In this study, topical pretreatment with AZD4547 was associated with a reduction in expression (mRNA) of proinflammatory cytokines and chemokines and also inhibited the dermal infiltration of proinflammatory mast cells and macrophages (Supplementary Figure S6). AZD4547 also reduced the mRNA expression of NfκB and COX2. These effects of AZD4547 likely also contributed to the inhibition of UVB-induced epidermal proliferation and UVB-induced skin carcinogenesis. In these studies, we found that topical AZD4547 (400 μg) in the absence of UVB led to modest but statistically significant increases in epidermal PDL1 and CCL2 mRNAs. Further work will be needed to determine the significance of these changes in mRNAs in terms of protein changes and any functional consequences.

As shown in Figure 6, topical treatment with AZD4547 significantly inhibited UVB-induced skin carcinogenesis. In this regard, the number of SCCs was significantly reduced, and tumor-free survival increased by AZD4547. Because AZD4547 is a pan-FGFR inhibitor, we cannot conclude which FGFR is most critical for its effects or whether all the three FGFRs detected in mouse epidermis may be involved. However, evidence for the role of FGFR2 in driving SCC development in a Le-Cre–driven PTEN null mouse model has been reported (Hertzler-Schaefer et al., 2014). In contrast, mice with constitutive, genetic deletion of Fgfr2 (K5-R2) using the K5-Cre/Lox system showed impaired skin barrier function and abnormal appendages, hair follicle growth, and cutaneous homeostasis. In addition, epidermal deletion of Fgfr2 sensitized animals to chemically induced skin papillomas and cSCC (Grose et al., 2007). However, it is important to note in this latter study that increased KC proliferation was
observed in these mice owing to progressive inflammation and not owing to cell-autonomous effects because K5-R2 KCs exhibited a standard rate of proliferation in vitro. Deletion of FGFR2 led to upregulation of FGFR1, which further confounds the results suggesting a protective role of FGFR2 in skin cancer development. In our earlier publication, UVB exposure induced FGFR2 activation in epidermal KCs in vitro and in vivo, and inhibition of FGFR using AZD4547 significantly decreased UVB-induced epidermal hyperproliferation. The results of these earlier studies are consistent with the current results and further support the role of FGFR2 in UVB skin carcinogenesis.

Finally, there are ongoing efforts to understand the role of VEGFRs in UVB-induced skin carcinogenesis (Hartono et al., 2022; Johnson and Wilgus, 2012). Although VEGFRs are mainly expressed on endothelial cells (Stefanini et al., 2009), the expression has also been observed in human KCs (Zhu et al., 2012). The expression of VEGFR2 in mouse epidermis remains controversial (Lichtenberger, 2010; Shahrabi-Farahani et al., 2014). Activation of phosphorylated VEGFR2 by UV exposure was shown to be time dependent in human KCs (Zhu et al., 2012). As shown in Supplementary Figure S5, phosphorylated VEGFR2 (Y1175) was elevated in the epidermis 4 hours after the last UVB exposure, and pretreatment with AZD4547 (400 μg) significantly reduced this phosphorylation. AZD4547 binds to FGFR1, 2, and 3 with higher affinity (half-maximal inhibitory concentration of 0.2, 2.5, and 1.8 nM, respectively); however, it also binds to VEGFR2 with lower affinity (half-maximal inhibitory concentration of 24 nM). Thus, inhibition of VEGFR by AZD4547 may also have contributed to the inhibitory effects observed on proliferation, inflammation, and SCC development. Further work will be necessary to determine the overall contribution of VEGFR inhibition to the effects of AZD4547 on UVB-induced cSCC formation.

In conclusion, we have established the involvement of FGFRs in UVB-induced skin carcinogenesis. In addition, we have shown that topical application of AZD4547 inhibited UVB skin carcinogenesis. Targeting specific FGFRs using topically applied agents could represent, to our knowledge, a previously unreported approach for prevention and/or treatment of cSCC.

**MATERIALS AND METHODS**

**Mice and short-term UVB irradiation**

All animal experiments were carried out in compliance with the institutional guidelines approved by the Animal Care and Use Committee at The University of Texas at Austin (Austin, Texas) and Louisiana State University Health (Shreveport, LA). Female FVB/N mice were procured from Charles River Laboratories (Wilmington, MA) and allowed to acclimatize for at least 1 week before experimentation. At ages 6–7 weeks, the dorsal skin of mice was shaved 48 hours before experiments for both control (no UVB) and UVB treatment groups. Mice were treated with UVB radiation (180 mJ/cm²) three times a week for 2 weeks on every other day. Mice were killed at different time points (2, 4, 6, 8, and 24 hours) after the last treatment, dorsal skin was removed, and the epidermis was scraped from the skin as previously described (Eguiarte-Solomon et al., 2021; Kataoka et al., 2008). Epidermal scrapings were pooled from four mice in each group.

**Short-term AZD4547 treatment**

Female FVB/N mice aged 6–7 weeks were pretreated with either acetone vehicle or AZD4547 (200 and 400 μg dissolved in 200 μl acetone) topically, 1 hour before UVB (180 mJ/cm²) exposure. Mice were treated with UVB radiation three times a week for 2 weeks every other day. Mice were killed after 4 hours of the last treatment, and the skin was divided into two halves. One half was used for histopathological analysis, and the other half was used for epidermal scrapings. The epidermal lysates were further used for protein and RNA extraction using 0.3% Chaps buffer and TRIZol, respectively.

**UVB-induced skin carcinogenesis**

For long-term UVB experiments elucidating the effect of AZD4547 on UVB-induced cSCC, female FVB/N mice (n = 10 per group, aged 6–8 weeks) were utilized. Briefly, each mouse’s dorsal skin was shaved 48 hours before UVB irradiation; only those mice in the resting phase of the hair cycle were utilized for the study. For UVB irradiation, Westinghouse FS20 sun lamp bulbs with peak emission at 313 nm were used. The fluence rate was measured with an IL1400A radiometer/photometer coupled to a SEL240/UVB-1/TD detector (International Light, Newburyport, MA). Mice were anesthetized using isoflurane (2%) at the time of UVB exposure. Mice were topically treated with either acetone (100 μl) or AZD4547 (400 μg/100 μl of acetone). One hour after the treatment, mice were either sham irradiated or exposed to UVB (1,200 J/m²). The paradigm was followed three times a week until the end of the study. The UVB dose was gradually increased by 25% every week, starting at 1,200 J/m² to a maximum of 4,500 J/m² until the end of the study. Mice were monitored for tumor formation weekly. Skin tumors developed on dorsal skin were counted after the first tumor appearance. After the termination of experiments, mice were killed, and tumors were collected for histopathologic analysis.

**Western blot analysis**

Protein was isolated from skin epidermal lysates as described earlier and subjected to western blot analysis as previously described (Eguiarte-Solomon et al., 2021; Kataoka et al., 2008). The following antibodies were used: p53 (32532S, Cell Signaling Technology, Danvers, MA), FGFR1 (9740S, Cell Signaling Technology), FGFR2 (ab109372), FGFR3 (ab133644), phosphorylated FGR (3471S, Cell Signaling Technology), phosphorylated FRS2 (3746S, Cell Signaling Technology), phosphorylated FRS2α (3861S, Cell Signaling Technology), p53 (ab137458), Akt (4056, Cell Signaling Technology), Akt (9272, Cell Signaling Technology), p53 (32532S, Cell Signaling Technology), STAT1 (9172L, Cell Signaling Technology), phosphorylated STAT1 (9167, Cell Signaling Technology), phosphorylated ERK1/2 (9101, Cell Signaling Technology), MAPK/ERK kinase (9102, Cell Signaling Technology), phosphorylated MAPK/ERK kinase 1/2 (9150, Cell Signaling Technology), phosphorylated p70S6K (9234, Cell Signaling Technology), p70S6K (9202S, Cell Signaling Technology), phosphorylated 4EBP1 (9246S, Cell Signaling Technology), 4EBP1 (9244S, Cell Signaling Technology), phosphorylated ERK (9102, Cell Signaling Technology), phosphorylated JNK (9251, Cell Signaling Technology), phosphorylated JNK (9251, Cell Signaling Technology), phosphorylated PI3K (3230, Cell Signaling Technology), phosphorylated PRAS40 (2691S, Cell Signaling Technology), phosphorylated PRAS40 (2997, Cell Signaling Technology), and Akt (4066, Cell Signaling Technology).

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Western blots were quantitated from at least three independent experiments using Mann–Whitney U test. The statistical analysis for selected proteins was performed using TRIzol reagent, and cDNA was prepared from the skin epidermal lysates. Extractions were performed using TRIzol reagent, and cDNA was prepared using the High-Capacity cDNA Reverse-Transcription Kits (Applied Biosystems, Waltham, MA) according to the manufacturer’s instructions. For RT-qPCR analysis, we used iTaq universal SYBR green supermix (Bio-Rad Laboratories, Hercules, CA). The RT-qPCR reactions were performed and analyzed on a Viia7 instrument (Applied Biosystems) using the comparative ΔΔCt method and normalized to the 18S housekeeping gene. Supplementary Table S1 lists the genes and primers used for the RT-qPCR analyses in this study.

Statistical analyses

A comparison of differences in labeling index, inflammatory cells, and UV photoproducts was performed using the Mann–Whitney U test. Multiple unpaired t-test was used for the comparisons of cumulative counts of SCCs in the UVB carcinogenesis experiments. For comparisons of Kaplan–Meier survival curves, we used the Log-rank (Mantel–Cox) test for statistical significance (Prism 5, GraphPad Software, San Diego, CA). For the RT-qPCR analyses, we used one-way ANOVA/Tukey’s test. The statistical analysis for selected protein levels from western blot analyses was performed with triplicates from at least three independent experiments using Mann–Whitney U test. Significance in all cases was set at P < 0.05.

Data availability statement

No datasets were generated or analyzed during this study.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: ARK, JD, CAON; Formal Analysis: MAT, ARK; Funding Acquisition: ARK, CAON, JD; Investigation: MAT, ARK, RA, ROK; Methodology: MAT, ARK, XG, OR, SC, RA; Project Administration: ARK, CAON, JD; Resources: CAON, JD; Supervision: ARK, CAON, JD; Visualization: MAT, ARK; Writing – Original Draft Preparation: MAT, ARK; Writing – Review and Editing: CAON, JD

Disclaimer

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2022.03.036

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Supplementary Figure S1. Graphical quantitation of western blots from Figure 1. Quantitation of protein changes in (a) FGFRs and (b) FGFs in the epidermis after exposure to UVB. Protein lysates were collected from mice skin exposed to UVB, three times a week for 2 weeks. Western blots were analyzed and quantified by normalizing with a loading Con. Asterisk (*) denotes significance ($P \leq 0.05$) compared with the sham group using Mann–Whitney U test. Con, control; FGF, fibroblast GF; FGFR, fibroblast GF receptor; pFGFR, phosphorylated fibroblast GF receptor.
Supplementary Figure S2. Epidermal FGF10 protein levels. Western blot analysis shows the time course of changes in FGF10 protein levels in the epidermis after exposure to UVB. Epidermal protein lysates were collected from the mouse skin exposed to UVB, three times a week for 2 weeks. Western blots were analyzed and quantified by normalizing with GAPDH as a loading Con. Con, control.
Supplementary Figure S3. Graphical quantitation of western blots from Figure 2. Quantitation of epidermal protein changes after the last UVB exposure by normalizing phosphorylated proteins to both loading control (vinculin or actin) and respective total protein Con. (a) Akt/mTORC1 pathway, (b) STAT pathway, (c) MAPK pathway. Quantitation of blots represents the average from at least three independent experiments. Asterisk (*) denotes significance \( P \leq 0.05 \) compared with the sham group using Mann–Whitney U test. Akt, protein kinase B; Con, control; p4EBP1, phosphorylated 4EBP1; pAkt, phosphorylated protein kinase B; pERK, phosphorylated extracellular signal–regulated kinase kinase; plak, phosphorylated Jak; pMEK, phosphorylated MAPK/extracellular signal–regulated kinase kinase; p-P70S6K, phosphorylated P70S6K; p-PRAS40, phosphorylated PRAS40; p-rS6, phosphorylated r-S6; pSTAT, STAT, phosphorylated signal transducer and activator of transcription; STAT, signal transducer and activator of transcription.
Supplementary Figure S4. Topical treatment of AZD4547 did not have a photoprotective effect. Groups of mice (n = 4) were treated topically with acetone vehicle or AZD4547 1 hour before UVB radiation, and samples were collected 4 hours after the last treatment. (a) Representative CPD staining of the epidermis. (b) CPD-positive keratinocytes were counted microscopically (×20 magnification, bar = 100 µm) in at least three nonoverlapping fields in sections from each mouse and calculated as the percentage of photoproduct-positive cells per centimeter. ***P < 0.001 by one-way ANOVA. CPD, cyclobutane pyrimidine dimer.
Supplementary Figure S5. Protein levels of pVEGFR2. Groups of mice (n = 4) were treated topically with acetone vehicle or AZD4547 1 hour before UVB radiation, and skin epidermal lysates were collected 4 hours after the last treatment. Representative western blot shows a statistically significant increase in p-VEGFR2 after exposure to UVB. Topical AZD4547 (400 µg) pretreatment significantly inhibited UVB-induced phosphorylation of VEGFR2. The bar graph represents the phosphorylated protein level normalized to total protein and the loading control and presents the fold change compared with that of the respective acetone control group. Data represents n = 3 independent experiments (mean ± SEM). Significance (*P ≤ 0.05) was calculated using one-way ANOVA. pVEGFR2, phosphorylated VEGFR2.
Supplementary Figure S6. Effect of topically applied AZD4547 on UVB-induced proinflammatory gene expression. AZD4547 (400 μg) was topically treated 1 hour before UVB radiation (180 mJ/cm²), and epidermal lysate (pooled four mice per group) was collected after 4 hours of last treatment. Graphs show mRNA expression of Tnfα, Ccl2, Il1α, Cxcl9/10/11, Nfkβ, Pdl1, and Cox2 by qRT-PCR analyses. Graphs represent mean ± SEM (an average of at least three independent experiments), and significance (⁎P ≤ 0.05, ⁎⁎P < 0.01, ⁎⁎⁎P < 0.001, and ⁎⁎⁎⁎P < 0.0001) was calculated using one-way ANOVA.
### Supplementary Table S1. Genes and Primers Used for the qRT-PCR Analyses

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<th>Gene</th>
<th>PCR Primers</th>
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<td>TNFa</td>
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Abbreviations: Fw, forward; Rev, reverse.