Photopreventive Effect and Mechanism of AZD4547 and Curcumin C3 Complex on UVB-Induced Epidermal Hyperplasia
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Abstract
Aggressive cutaneous squamous cell carcinoma (cSCC) of the skin is the second most common type of skin cancer in the United States due to high exposure to ultraviolet B (UVB) radiation. In our previous studies, Curcumin C3 complex (C3), a standardized preparation of three curcuminoids, delayed UVB-induced tumor incidence and inhibited multiplicity. Exposure to UVB activates mTOR and FGFR signaling that play a key role in skin tumorigenesis. The purpose of this study was to investigate the efficacy of C3 complex to afford protection against acute UVB-induced hyperproliferation by targeting the mTOR and FGFR signaling pathways. Pretreatment with C3 complex significantly inhibited UVB-induced FGF-2 induction, FGF-2–induced cell proliferation, progression and colony formation, mTORC1 and mTORC2 activation, and FGFR2 phosphorylation in the promotion-sensitive JB6 cells epithelial cells. Further, FGFR was critical for UVB-induced mTOR activation, suggesting an important role of FGFR2 in UVB-induced mTOR signaling. SKH-1 mice pretreated with C3 (15 mg/kg/b.w.) for 2 weeks followed by a single exposure to UVB (180 mj/cm²) significantly attenuated UVB-induced mTORC1, mTORC2, and FGFR2 activation. To further assess the role of FGFR in UVB-induced hyperproliferation, SKH-1 mice were pretreated with AZD4547 (5 mg/kg/b.w.), a selective pan-FGFR kinase inhibitor followed by single exposure to UVB (180 mj/cm²). AZD4547 significantly inhibited UVB-induced mTORC1 and mTORC2 activation, epidermal hyperplasia and hyperproliferation. Our studies underscore the importance of FGFR signaling in UVB-induced acute skin changes and the role of FGFR/mTOR signaling in mediating the effects of C3 complex in the pathogenesis of skin cancer. Cancer Prev Res; 9(4); 296–304. ©2016 AACR.

Introduction
According to the American Cancer Society, skin cancer is the most common of all cancers and accounts for nearly half of all cancers in the United States (1, 2). Non-melanoma skin cancer (NMSC) is the most common cancer in the United States, with over 3.5 million new cases of the two most common forms, squamous cell carcinoma (SCC) and basal cell carcinoma (BCC), anticipated annually (3). The more clinically aggressive form, SCC of the skin (cSCC), has been increasing in incidence since the 1960s, and the annual rate has reached 10% in recent years. This surge is a result of increased exposure to sunlight (sunbathing) or use of sunbeds and increased UV content of sunlight due to ozone depletion (4). High-risk populations include people with fair complexion as well as sun-damaged skin, actinic keratosis (AKs; benign or premalignant lesions, a.k.a. solar keratosis), and resected premalignant or malignant skin cancers (5). cSCC comprises ~16% to 20% of all skin cancers; it is highly invasive and could metastasize if neglected, necessitating the need for photo-preventive agents. C3 complex, collectively known as curcuminoids, is a standardized preparation (Sabinsa) of three curcuminoids—curcumin (76.07%), demethoxycurcumin (DMC, 20.28%), and bisdemethoxycurcumin (3.63%). The safety and efficacy of C3 complex is established in clinical trials (6–8). DMC and bisdemethoxycurcumin, natural demethoxy derivatives of curcumin, possess anti-inflammatory, and antiproliferative properties and are more stable than curcumin in serum (9). Curcumin has been investigated as a chemo preventive agent in a variety of cancers, such as pancreatic, colon, liver, hematologic, and oral cancers (10, 11). Repetitive exposure to UVB induces mutations as well as activates signaling pathways, thus acting as a tumor initiator as well as promoter (12, 13). In our published studies, we established that C3 complex inhibits UVB-induced tumor incidence and multiplicity (14). However, the mechanism for its photo-preventive effects is unclear and its molecular mechanism has yet to be clearly defined.
Curcumin is a membrane disruptor and activates a wide array of surface receptors. Among these, FGFRs are transmembrane receptor tyrosine kinases (RTK) of the immunoglobulin (Ig) superfamily. In humans, the FGFR family consists of 4 genes (FGFR1–FGFR4) encoding closely related transmembrane RTKs (15, 16). Binding of FGFs to FGFRs induces receptor dimerization, leading to conformational changes that enable trans-phosphorylation of tyrosines in the intracellular domain, including the kinase...
domain and the C-terminus (17). Phosphorylation of FGFR activates multiple intracellular signaling cascades, including Ras–RAF–mitogen–activated protein kinase, MEK–ERK and PI3/AKT pathway. FGF signaling is required for epidermal growth, skin barrier function, and hair cycle activation (18). However, inappropriate induction of FGF/FGFR signaling promotes skin papillomas, which can be reversed by the epidermal-specific Fgfr2 ablation (19). Upregulation of Fgf2, Fgf7, Fgf10, and Fgf22, and subsequent activation of Fgfr2 signaling, has been implicated in aberrant keratinocyte proliferation in diseases, such as acne and psoriasis (20). On the other hand, epidermal deletion of Fgfr2 sensitized animals to chemically induced skin papillomas and SCCs (19). Therefore, the role of FGF signaling in skin homeostasis and tumorigenesis is highly context dependent. Accordingly, we sought to determine the photo-preventive effects of C3 complex and FGFR inhibitor (AZD4547) via the FGFR2-dependent mechanism. Curcumin inhibits mTOR signaling, an essential serine/threonine kinase responsible for cell survival and proliferation (21). mTOR forms two functionally distinct complexes: mTORC1 and mTORC2. mTORC1 is rapamycin-sensitive, while mTORC2 is rapamycin resistant with S6K and AKT as downstream effectors, respectively (22). Interestingly, recent studies have demonstrated the FGF signaling axis to activate the mTOR pathway (23). Further, dual inhibitors for mTOR and FGFR inhibit tumor growth in the orthotopic model (24). Accordingly, we explored the UVB-induced, FGF-mediated activation of mTOR signaling in the skin and established the potential role of C3 in mediating its effects via the FGFR/mTOR mechanism.

Materials and Methods

Cell line, reagents, and treatment

Murine epidermal JB6 P+ cells were a kind gift from Dr. Zhao (LSU Health Shreveport, Shreveport, LA; ref. 25). Please note that the cell lines were not authenticated by the authors. The cells were grown and maintained in Essential Medium (EMEM) supplemented with 5% FBS, 2 mmol/L of L-glutamine, 100 U/mL penicillin, and 50 μg/mL streptomycin. Curcumin C3 complex was obtained from Sabinsa Corporation. C3 complex (10 μmol/L) was dissolved in ethanol and used for the cell culture experiments. For in vivo studies, C3 was dissolved in corn oil and administered orally as indicated. AZD4547, a selective FGFR inhibitor targeting FGFR1/2/3 (Selleckchem), was dissolved in 50% ethanol and 50% ethanol containing dimethyl sulfoxide. Curcumin C3 complex was obtained from Sabinsa Corporation. C3 complex (10 μmol/L) was dissolved in ethanol and used for the cell culture experiments. For in vivo studies, C3 was dissolved in corn oil and administered orally as indicated. AZD4547, a selective FGFR inhibitor targeting FGFR1/2/3 (Selleckchem), was dissolved in 50% ethanol and 50% ethanol containing dimethyl sulfoxide. All animal protocols were approved in advance by the Institutional Care and Use Committee at LSU Health Shreveport. In accordance with the policies and guidelines set forth by the Office of Laboratory Animal Welfare and the NIH. Female SKH-1 mice (4–6 weeks, The Jackson Laboratory) were used for experiments. The dorsal surface of SKH-1 mice was pretreated with C3 complex (15 mg/kg/b.w.) or vehicle (corn oil) for 5 days a week for 2 weeks. At the end of 2 weeks, mice were exposed to a single dose of UVB (180 mJ/cm²). At 24 hours after exposure, mice were sacrificed, and whole skin was collected. Blood was collected using cardiac puncture at 15,000 x g to isolate the plasma for the determination of FGF-2 using ELISA. For studies determining the effects of FGFR inhibition on UVB-induced acute skin changes, mice were pretreated with AZD4547 (5 mg/kg/b.w.) for 2 weeks followed by a single exposure to UVB (180 mJ/cm²) and processed as indicated above.

Cell transfection

Wild-type (WT) and constitutively active mutants for FGFR2 (FGFR2-WT pCDNA3 and FGFR2-C278F pCDNA) expression plasmids were obtained from Dr. Donghwee at University of California (San Diego, CA). JB6 P+ cells were either mock transfected or transfected with FGFR2-pCDNA3 using Lipofectamine 3000 (Invitrogen). Transfection efficiency was confirmed using Western blot analysis with FGF2 (Santa Cruz Biotechnology, 1:1,000).

UVB treatment

JB6 cells were seeded at a confluence of 70% in 60-mm tissue culture dishes in DMEM containing 5% FBS. Cells were starved overnight in 0.1% FBS and treated as indicated. For exposure to UVB (50 mJ/cm²), FS20 UVB bulbs, National Biological, emitting UV light between 290 to 320 nm), cells were washed with PBS and then exposed to UVB in minimal volume of PBS just sufficient to layer the cells. Media were added back to the cells after exposure. Cells were lysed at given time points and subjected to experimental analysis as indicated.

Cell viability and cell cycle

Cells were treated as indicated, and cell viability was assessed using the MTS assay (CellTiter 96 Aqueous Proliferation Assay, Promega). For cell-cycle analysis, cells were synchronized overnight in 0.1% FBS containing media. Cells were treated as indicated and stained with Ki67 stain and analyzed using flow cytometry (26).

Soft-agar assay

Anchorage-independent colony formation was assessed using soft-agar assay and performed as described by Zhao and colleagues (25). The bottom agar was prepared by adding desired treatments (e.g., 10 μmol/L C3 complex) to the 0.5% agar mix. The top agar layer was prepared by diluting 1 fraction of 1 × 10⁵ cells/mL in a single-cell suspension with two fractions of 0.5% agar mix and desired treatments. Cells were incubated in a humidified 37°C, 5% CO₂ incubator for 2 weeks, stained by adding tetrazolium dye that produces water-soluble formazan, and absorbance was measured.

ELISA

Cell media, plasma samples, and tissue lysates were analyzed for FGF-2 (Promega) and FGFR2 phosphorylation (Cell Signaling Technology) using an ELISA kit as per the manufacturer’s instructions. mTORC1 and mTORC2 levels were detected by measuring the downstream effectors p70S6K and pAKT using p70S6K ELISA Kit Simple Step (pT389 + total) and AKT1/2/3 pS473 + AKT1 ELISA Kit (Abcam).

Tissue processing and Western blot analysis

Frozen skin samples were homogenized in RIPA buffer using a Polytron homogenizer followed by centrifugation at 30,000 x g for 30 minutes at 4°C, and supernatants were collected. Western blot analysis was conducted using gel electrophoresis. Antibodies used include AKT, p-AKTSer473, S6K, p-S6KThr389, actin (all
from Cell Signaling Technology, 1:1,000). Data were normalized using actin. In experiments involving measurement of pFGFR2, mTORC1, and mTORC2, tissue homogenate supernatant was collected as described above and subjected to ELISA for pFGFR2, mTORC1 (pS6K/S6k), and mTORC2 (pAKT/AKT; all from Abcam).

Histologic analysis

Skin tissues were processed and embedded in paraffin, and 5-µm sections were cut for immunohistochemistry (IHC) and hematoxylin and eosin (H&E) staining for histopathologic examination. H&E-stained sections were assessed for epidermal hyperplasia by measuring epidermal thickness at 4 different regions for each mouse section. Sections were subjected to IHC analysis for FGFR2 and Ki67 (Santa Cruz Biotechnology, 1:200) for determination of cell proliferation. The number of Ki67+ cells was quantified by determining the total number of Ki67+ cells per ×20 magnification for five areas randomly selected (27).

Immunohistochemistry for FGFR2 in SCC patients

An IRB approved study was performed to identify patients with cSCC at LSU Health-Shreveport and Overton Brooks Veterans Affairs Medical Center. Normal adjacent skin and SCC sections (n = 21) were immunostained for FGFR2 (1:600, Santa Cruz Biotechnology) using DAB as a chromogen, and the staining intensity was quantified by a registered pathologist who was blinded to the clinical details. A semi-quantitative approach was used to score the staining intensity. Slides were categorized based on the following: no staining scored as (0), weak or focal staining scored as (1+-), moderate staining scored as (2+), and strong staining scored as (3+). The H score method was utilized, and the percent positivity was reported based on predominant staining intensity.

Statistical analysis

Data are expressed as the mean of at least 3 independent experiments and analyzed by either ANOVA followed by the Tukey post hoc test or two-sided Student t test, when applicable (Graph Pad Prism 6). A P value of <0.05 was considered statistically significant.

Results

C3 inhibits FGF-2–induced JB6 cell proliferation

Exposure to lower doses of UVB induces proliferation of the epidermal keratinocytes in the skin, leading to epidermal hyperplasia (28). We first established the role of FGF-2 in UVB-induced acute damage by exposing JB6 cells to sub-apoptotic dose of UVB (50 mj/cm²). As shown in Fig. 1A, exposure of JB6 cells to UVB (50 mj/cm²) significantly increased FGF-2 levels in the media at 24 hours after UVB irradiation. Pretreatment of cells with C3 (10 µmol/L) for 1 hour significantly decreased UVB-induced FGF-2 levels in the media. It is well established that hyperproliferation of epidermal keratinocytes leads to tumor promotion. Accordingly, we assessed the inhibitory effects of C3 on FGF-2–induced JB6 cell proliferation. Cells were pretreated with C3 for 1 hour followed by stimulation with FGF-2 (10 ng/mL). As shown in Fig. 1B, pretreatment with C3 significantly inhibited FGF-2 induced JB6 proliferation at 24 hours. To further determine the efficacy of C3 in inhibiting FGF-2–induced tumor promotion activity, JB6 cells were pretreated with C3 and subjected to FGF-2–induced cell-cycle progression and anchorage-independent growth and tumorigenic phenotype assay. As shown in Fig. 1C and D, FGF-2 stimulation increased the total number of cells in the S phase at 24 hours after stimulation and promoted colony formation in the soft-agar assay at the end of 2 weeks. Pretreatment with C3 significantly inhibited FGF-2–induced, S phase cell traverse and colony formation.

C3 inhibits UVB and FGF-2–induced mTORC1 and mTORC2 activation in JB6 cells

Cellular proliferation is a key event in the pathogenesis of UVB-induced photo-carcinogenesis. In addition to tumor-initiating effects, exposure to UVB leads to tumor promotion via mitogenic and survival signaling. Accordingly, we sought to determine the mechanism for inhibitory effects of C3 on UVB-induced JB6 proliferation. The effect of C3 complex on two different mitogens and survival factors (UVB and FGF-2) and their signaling pathways was studied. Exposure to UVB activates mTOR survival pathways (22). However, the effect of C3 on UVB-induced mTOR and FGFR signaling is not well studied. We investigated the effects of C3 on UVB-induced mTORC1 and mTORC2 activation by measuring phosphorylation of S6K and AKT (Ser473), the downstream target of mTORC1 and mTORC2, respectively. As shown in Fig. 2A and B, low dose of UVB (50 mj/cm²) and FGF-2 (10 ng/mL) activates both mTORC1 and mTORC2 signaling pathways as measured by phosphorylation of S6K and AKT(Ser473), respectively. The activation of mTORC1 and mTORC2 was accompanied with an increase in FGFR2 expression measured by Western blot analysis (Fig. 2C). Pretreatment with C3 complex abrogated UVB and FGF-2–induced mTOR activation. Interestingly, C3 attenuated UVB and FGF-2–induced increase in expression of FGFR2 (Fig. 2C). This suggests a potential role of FGFR2 in modulating C3-mediated effects on UVB-induced mTOR signaling. Overexpression of WT and constitutively active FGFR2 using FGFR2-WT cDNA and FGFR2-C278F significantly increased S6 and AKT phosphorylation, suggesting that FGFR2 acts upstream in the signaling mechanism (Fig. 2D). Finally, to determine whether UVB-induced S6K phosphorylation and mTOR activation require FGFR, JB6 cells were pretreated with a selective FGFR kinase inhibitor (AZD4547) followed by sub-apoptotic dose of UVB. As shown in Fig. 2E, pretreatment with AZD4547 significantly inhibited UVB-induced S6K and AKT phosphorylation, suggesting the importance of FGFR signaling in UVB-induced effects of mTOR signaling (Fig. 2C).

C3 inhibits UVB-induced epidermal hyperproliferation and hyperplasia

To further translate our in vitro findings in an in vivo mouse model of photo-carcinogenesis, SKH-1 mice were pretreated with C3 (15 mg/kg/b.w.) for 2 weeks and were subjected to a single dose of UVB exposure (180 mj/cm²). After 24 hours, mouse skins were harvested and analyzed. Consistent with our in vitro studies, pretreatment with C3 significantly inhibited UVB-induced epidermal hyperplasia and hyperproliferation (Fig. 3A and B and Fig. 3C and D). To further bolster our in vitro data, UVB increased FGF-2 in the epidermis, compared with sham-irradiated control (Fig. 3E). C3 did not affect baseline FGFR2 phosphorylation levels in control skin, but restored FGFR2 expression to the low, control levels in the UVB-exposed mouse skin (Fig. 3F).

The decrease in UVB-induced FGFR2 expression by C3 was accompanied by inhibition of mTORC1 (Fig. 3G) and mTORC2 (Fig. 3H) activation and decrease in epidermal hyperplasia.
Additionally, C3 also attenuated serum FGF-2 levels (Supplementary Fig. S1) in UVB-exposed mice compared with vehicle-treated controls. This suggests both local and systemic efficacy of C3, which should ensure especially significant protection from UVB damage.

FGFR inhibition attenuates UVB-stimulated epidermal hyperplasia and hyperproliferation

Overexpression of FGFR is associated with the progression of variety of epithelial cancers. FGFR plays an important role in normal morphogenesis and tissue repair, including DNA synthesis, proliferation, and migration of epithelial cells (18). Accordingly, we first sought to investigate the role of FGFR in FGF2–induced cell proliferation. JB6 cells were synchronized by serum starvation for 24 hours. Cells were pretreated with C3 (10 μmol/L) for 1 hour followed by stimulation with FGF-2 (10 ng/mL). Cell proliferation was measured at the end of 24 hours using the MTT assay. Data, mean ± SEM for n = 3 each group. *, P < 0.05, compared with control and C3 group. #, P < 0.05, compared with the UVB group. B, C3 inhibits FGF-2–induced cell proliferation. JB6 cells were synchronized by serum starvation for 24 hours. Cells were pretreated with C3 (10 μmol/L) for 1 hour followed by stimulation with FGF-2 (10 ng/mL). Cell proliferation was measured at the end of 24 hours using the MTT assay. Data, mean ± SEM for n = 3 each group. *, P < 0.05, compared with control and C3 group. #, P < 0.05, compared with the UVB group.

C, cell-cycle analysis of S-phase analyzed by flow cytometry using Ki67 stain 24 hours following FGF-2 (10 ng/mL) exposure. D, C3 inhibits FGF-2–induced anchorage-dependent cell transformation. JB6 cells were treated with either vehicle or C3 complex (10 μmol/L) together with FGF-2 and placed in the top agar layer onto the bottom agar layer as described in Materials and Methods. At the end of 2 weeks, colony growth was observed, stained and absorbance measured. Data, mean ± SEM for n > 3 experiments. *, P < 0.05, compared with vehicle control and FGF-2 group respectively.

Figure 1.
A, UVB induces FGF-2. JB6 cells were pretreated with either vehicle or C3 complex (10 μmol/L) for 1 hour followed by exposure to sub-apoptotic doses of UVB (50 mj/cm²). Cell culture media were collected at 24 hours, and FGF-2 levels were measured using an ELISA. Data, mean ± SEM, FGF-2 levels (pg/mL) for n > 3 experiments. *, P < 0.05, significance compared with sham control and C3 group. #, significance compared with the UVB group. B, C3 inhibits FGF-2–induced cell proliferation. JB6 cells were synchronized by serum starvation for 24 hours. Cells were pretreated with C3 (10 μmol/L) for 1 hour followed by stimulation with FGF-2 (10 ng/mL). Cell proliferation was measured at the end of 24 hours using the MTT assay. Data, mean ± SEM for n = 3 each group. *, P < 0.05, compared with control and C3 group. #, P < 0.05, compared with the UVB group. B, C3 inhibits FGF-2–induced cell proliferation. JB6 cells were synchronized by serum starvation for 24 hours. Cells were pretreated with C3 (10 μmol/L) for 1 hour followed by stimulation with FGF-2 (10 ng/mL). Cell proliferation was measured at the end of 24 hours using the MTT assay. Data, mean ± SEM for n = 3 each group. *, P < 0.05, compared with control and C3 group. #, P < 0.05, compared with the UVB group. C, cell-cycle analysis of S-phase analyzed by flow cytometry using Krishans stain 24 hours following FGF-2 (10 ng/mL) exposure. D, C3 inhibits FGF-2–induced anchorage-dependent cell transformation. JB6 cells were treated with either vehicle or C3 complex (10 μmol/L) together with FGF-2 and placed in the top agar layer onto the bottom agar layer as described in Materials and Methods. At the end of 2 weeks, colony growth was observed, stained and absorbance measured. Data, mean ± SEM for n > 3 experiments. *, P < 0.05, compared with vehicle control and FGF-2 group respectively.

Additionally, C3 also attenuated serum FGF-2 levels (Supplementary Fig. S1) in UVB-exposed mice compared with vehicle-treated controls. This suggests both local and systemic efficacy of C3, which should ensure especially significant protection from UVB damage.

FGFR inhibition attenuates UVB-stimulated epidermal hyperplasia and hyperproliferation

Overexpression of FGFR is associated with the progression of variety of epithelial cancers. FGFR plays an important role in normal morphogenesis and tissue repair, including DNA synthesis, proliferation, and migration of epithelial cells (18). Accordingly, we first sought to investigate the role of FGFR in FGF2–induced cell-cycle progression and proliferation using AZD4547. Consistent with prior studies, treatment of JB6 cells with FGF-2 significantly induced JB6 proliferation (Fig. 4A). Pretreatment with AZD4547 significantly inhibited FGF-2–induced JB6 proliferation (Fig. 4A) at 24 hours after stimulation. It was previously shown that FGF-2 activates FGFR to induce G1–S cell-cycle progression in serum-deprived JB6 cells and also promotes anchorage-independent colony formation (29). Accordingly, to determine whether FGFR contributes to cell-cycle progression and anchorage-independent growth, JB6 cells were serum-starved to synchronize them in G0 phase and pretreated with AZD4547 for 1 hour followed by FGF-2 exposure for 24 hours. FGF-2–stimulated cell-cycle progression in vehicle-treated cells, as measured by the percentage of cells in S-phase at 24 hours after exposure. Pretreatment with AZD4547 significantly decreased the proportion of cells in the S phase (Fig. 4B). Additionally, at the end of 2 weeks, AZD4547 decreased FGF-2–induced colony formation (Fig. 4C). As shown earlier in Fig. 4C, the decrease in FGF-2–induced cell proliferation was accompanied with an inhibition mTORC1 and mTORC2, and FGFR activation (Fig. 2C). We further investigated the effect of FGFR deficiency in UVB-induced epidermal hyperplasia (as measured by epidermal thickness) and epidermal proliferation (as measured by Ki67+ IHC) in vivo using AZD4547.
Consistent with our prior \textit{in vitro} data, UVB irradiation significantly increased epidermal hyperplasia (Fig. 5A and B) and epidermal proliferation (Fig. 5C and D) 24 hours following UVB exposure. AZD4547 alone had no effect on epidermal thickness or proliferation in mock-irradiated animals. Pretreatment of SKH-1 mice with AZD4547 for 2 weeks significantly inhibited UVB-induced FGFR2 activation (pFGFR2; Fig. 5E). Further, AZD pretreatment attenuated UVB-induced epidermal hyperplasia and proliferation (Fig. 5B and D). Collectively, these results suggest that FGFR plays an important role in epidermal hyperproliferation and keratinocyte cell-cycle progression following UVB irradiation. To determine the effect of AZD on mTOR signaling \textit{in vivo}, total skin lysates were subjected to immunoblotting. As shown in Fig. 5E, pretreatment with AZD4547 significantly inhibited UVB-induced S6K and AKT phosphorylation, suggesting a direct role of FGFR in modulating mTOR signaling (Fig. 5F and G).

\textbf{Discussion}

This study with C3 complex has resulted in the identification of a novel FGFR/mTOR signaling pathway for the effective prevention of UVB-induced skin cancer. The role of FGFR/mTOR signaling should be further validated as a prospective photo-prevention target in skin cancer. Recently, FGF/FGFR signaling has attracted considerable attention for its critical role in endometrial, breast, hepatocellular, gastric, and pancreatic cancer via modulating cell survival, proliferation, and differentiation (30–34). AZD4547, a pan-FGFR inhibitor, is an exciting novel targeted agent shown to provide efficacy in several phase II clinical trials.

\textbf{Increase FGFR2 in patients with SCC}

In order to establish evidence for the role of FGFR2 in the pathogenesis of cutaneous SCC, we identified 21 patients with cSCC and immunostained the tumors for FGFR2 and compared them with adjacent normal skin. As shown in Fig. 6, FGFR2 staining was weakly distributed throughout the epidermis in the normal skin samples. Patients with SCC showed increased staining intensity in the tumor compared with normal skin, suggesting that FGFR2 is upregulated in cSCC. Tumors are heterogeneous, and thus the percentage of tumor stained with the highest staining intensity pattern was analyzed. Out of 21 patients, 8 patients exhibited 2–3+ positivity for less than 30% of tumor while 13 patients were 2–3+ positive for more than 50% of the tumor tissue immunostained for FGFR2.

\textbf{Figure 2.}

C3 inhibits UVB and FGF-2–induced mTOR signaling. JB6 cells were synchronized by serum starvation, pretreated with C3 (10 µmol/L) for 1 hour followed by exposure to either UVB (50 mj/cm²) or FGF-2 (10 ng/mL). A and B, at the end of 30 minutes, cells were lysed and analyzed for mTORC1 and mTORC2, respectively, using ELISA. *, significance compared with vehicle group; #, significance (p<0.05) compared with FGF-2 and UVB group, respectively. C, FGFR2 expression was assessed using Western blot analysis. Data were normalized to actin as a loading control. Representative blots for 3 experiments are shown. D, FGFR2 activates mTOR and mTOR2. JB6 cells were transfected with FGFR2-WT-cDNA and FGFR2-C278F mutants, and the effect on pS6 and pAKT activation was assessed using Western Blotting. E, UVB induces mTOR activation via FGFR. Cells were pretreated with AZD4547 for 1 hour followed by a single exposure to UVB (50 mj/cm²), and the effect of UVB on phosphorylation of S6K and AKT at the end of 30 minutes was evaluated by Western blot analysis. Representative blots for 3 experiments are shown.
C3 Complex and AZD4547 on UVB-Induced Epidermal Hyperplasia

Figure 3.
C3 inhibits UVB-induced epidermal hyperplasia and hyperproliferation in vivo. SKH-1 mice at 12 weeks of age were administered orally either C3 (15 mg/kg/b.w.) or vehicle for 14 days, before exposing to a single dose of UVB (180 mj/cm²) or mock irradiation. A, representative H&E images of skin sections obtained from mock (vehicle-treated), C3 (15 mg/kg/b.w.), UVB (180 mj/cm²), and C3+UVB groups. B, quantification of epidermal thickness (mean ± SEM) for 5 mice in each group. *, P < 0.05, compared with mock group. #, significance compared with UVB group. C, representative Ki67-stained images positively stained nuclei (Vector Red Chromogen). D, quantification of Ki67 cell proliferation index for n = 5 in each group. E, assessment of FGF-2 staining in mouse epidermis using IHC analysis and DAB as a chromogen showing positive stained nuclei in UVB-exposed mouse epidermis. F–H, analysis of pFGFR2, mTORC2, and mTORC1 activation markers in whole-skin extract harvested 24 hour following UVB radiation exposure, respectively. Whole skin tissue was lysed and subjected to ELISA for the measurement of pFGFR2, mTORC1, and mTORC2.

Figure 4.
Effect of AZD4547 on JB6 cell culture. A, AZD4547 inhibits FGF-2-induced cell proliferation; B, S phase progression; C, anchorage-independent growth. JB6 cells were synchronized by incubating overnight in media containing 0.0% FBS. Cells were pretreated with AZD4547 for 1 hour followed by exposure to FGF-2 (10 ng/mL). Proliferation was measured using the MTT assay at the end of 24 hours. For cell-cycle analysis, cells were synchronized, pretreated with AZD4547 for 1 hour followed by exposure to FGF-2 (10 ng/mL) for 24 hours. Cells were fixed, stained with Kristian stain, and subjected to flow cell-cycle measurement. For anchorage-independent growth assay, cells were pretreated for 1 hour followed by FGF-2 (10 ng/mL) exposure for 2 weeks. Values represent ± SEM for ≥3 experiments. *, significance compared with vehicle and AZD4547-alone group. #, significance compared with FGF-2 group with P < 0.05.
nuclear regulator of cell proliferation (42). FGF-2 binds and phosphorylates FGFR2 on serine 779 (S779) and activates downstream signaling via FGFR2 substrate (FRS2) and phospholipase C (PLC)

gamma (PLCgamma), leading to activation of STAT, Ras-dependent and Ras-independent MAPK pathway and P13–AKT signaling (43). Importantly, phosphorylation at serine 779 also controls cell proliferation and survival (43). In our studies, both WT and constitutively active FGFR2 pcDNA significantly increased mTORC1 and mTORC2 activation, which was ligand independent. Further, the effect on mTORC1 and mTORC2 appeared to be independent of FGFR2’s kinase activity. This is the first study providing direct evidence of the interaction between FGFR and mTOR signaling. AZD4547 is a specific pan-FGFR inhibitor and could potentially mediate its effects via the other FGFR subtypes FGFR3 and FGFR4. However, compared with other FGFR subtypes, FGFR2 is predominantly expressed in the epithelial cells. Deficiency of FGFR2 sensitizes animals to chemical-induced skin papillomas and SCC, thus acting as an oncogene (19). However, FGF signaling is also required for normal skin function and homeostasis, and activation of FGFR2 leads to keratinocyte proliferation in acne, psoriasis, and wound healing (20, 44). Further, FGFR2 is well established as a tumor promoter in sensitive JB6 cells to induce cell proliferation, cell-cycle transformation, and neoplastic cell changes (29). Tumor promotion is characterized by cell proliferation and cell-cycle progression that lead to the clonal expansion of initiated cells. Our studies demonstrated that both C3 complex and FGFR inhibitor decreased cell proliferation and cell-cycle progression, resulting in decreased epidermal hyperplasia and hyperproliferation in JB6 cells and UVB-irradiated SKH-1 mice in vivo.

To better understand the possible role of FGFR2 in the pathology of SCC, human skin SCC biopsies were stained for FGFR2 using IHC. Consistent with previous published reports, FGFR2 was significantly upregulated in patients with SCC compared with normal skin (45). FGFR2 gene amplification is often associated with FGFR2 overexpression and is reported in 10% of gastric cancer patients (46). Inhibition of FGFR2 inhibits the growth of FGF-receptor 2–driven gastric cancer models in vitro and in vivo. The role of mTOR1 and mTOR2 in UVB-induced skin cancer is well characterized by Carr and colleagues (22). Exposure to UVB activates mTORC1 and mTORC2 in the skin to modulate proliferation and apoptosis. In epidermal tumors, SCC and precancerous actinic keratoses, mTOR and its downstream effectors 4EBP1, S6K, are phosphorylated at much higher levels compared with...
normal skin. Furthermore, UVB-induces phosphorylation of 4EBP1, S6K, and AKT, suggesting an important role of the mTOR pathway in tumor initiation and progression (47). In our studies, pretreatment with C3 complex and FGFR inhibitor attenuated UVB-induced mTORC1 and mTORC2 activation. Through our pathway in tumor initiation and progression (47). In our studies, 4EBP1, S6K, and AKT, suggesting an important role of the mTOR signaling network induced by mTOR is essential for skin tumorigenesis (18). FGF signaling is activated by Pten–PI3K–mTOR signaling in the skin, suggesting an important cross-talk between the FGFR and mTOR signaling pathways. The oncogenic role of FGFR2 in SCC was further confirmed using genetic manipulation of FGFR2. In VSMCs, FGFR/FRS2 complex activates Akt/mTOR signaling to regulate gene expression (50). It is possible that FGFR2 activates mTORC1 and mTORC2 via its interaction with FRS2 and subsequent activation of tumor suppressor PTEN. Pten negatively regulates the PI3K-mTOR pathway and curcumin the parent component of C3 complex is well established to inhibit PTEN (51). However, further studies are required to identify whether the effects of C3 on UVB-induced mTOR activation are PTEN-dependent. Recent studies have also highlighted the importance of combined targeting of mutant FGFR2 and mTOR-driven signaling as a therapeutic strategy. Concomitant inhibition of FGFR2 and mTOR signaling by ponatinib and ridaforolimus resulted in synergistic antitumor activity in endometrial cancer (24). Our studies demonstrate Curcumin C3 complex inhibited both mTOR and FGFR2 signaling potentially leading to a new therapeutic strategy for advanced cancer with dual pathway dysregulation. Nevertheless, owing to its pleiotropic signal transduction profile, it is highly unlikely that C3 mediates all of its effects via the FGFR receptor system. In summary, our studies with C3 complex have resulted in establishing a critical role of FGFR in UVB-induced acute skin changes via the mTOR pathway.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
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References


Figure 6.
Increased FGFR2 staining in SCC. Normal skin epithelium shows weak brown trans-epithelial staining for FGFR2 compared with intense staining in patients with cSCC (DAB chromogen). All the patients with cSCC (n = 20) showed significantly higher staining scores for the SCC sections compared with normal adjacent skin. Representative FGFR2 IHC-stained images from patients with cSCC compared with normal adjacent skin are shown.


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