Topical kinase inhibitors induce regression of cutaneous squamous cell carcinoma

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Abstract
Actinic keratoses (AKs) and squamous cell carcinoma in situ (SCCIS) are precursor lesions for cutaneous squamous cell carcinoma (cSCC), the second most common form of cancer. Current topical therapies for AKs and SCCIS promote skin inflammation to eradicate lesions and do not directly target the biological mechanisms driving growth. We hypothesized that topical small molecule inhibitors targeting kinases promoting keratinocyte growth in AKs and SCCIS could induce regression of these lesions with less inflammation. To test this hypothesis, we determined the efficacy of topical dasatinib, 5-fluorouracil and BEZ-235 in inducing regression of cSCCs in the K14-Fyn Y528 transgenic mouse model. Topical dasatinib induced regression of cSCC with less inflammation, no ulceration and no mortality compared to 5-fluorouracil. Topical BEZ-235 induced cSCC regression similar to dasatinib without erythema or ulceration. These data indicate that topical small molecule kinase inhibitors targeting drivers of AK/SCCIS/cSCC growth represent a promising therapeutic approach to treat these common skin lesions.

1 | BACKGROUND

Cutaneous squamous cell carcinoma (cSCC) is the second most common cancer, with approximately 700,000 new lesions annually in the United States, incurring treatment costs approximating 1.6 billion dollars.1 cSCC incidence is expected to rise in an active, aging population increasing the need for new therapeutic strategies.2 Since cSCCs typically arise from actinic keratoses (AKs) and squamous cell carcinoma in situ (SCCIS), effective treatment of these precursor lesions could decrease the number of cSCCs.3 Current treatments for AKs/SCCIS include cryotherapy, curettage or excision which leads to skin dyspigmentation and/or scarring at significant financial cost. Topical therapies for AKs/SCCIS include 5-fluorouracil (5-FU), imiquimod, diclofenac and ingenol mebutate. 5-FU and imiquimod produce prominent inflammation limiting compliance, and diclofenac has demonstrated suboptimal clearance rates.4,5 Ingenol mebutate is irritating, promotes endothelial cell necrosis and is costly compared to other therapies.6,7 Therefore, there is a medical need for effective topical AK therapeutics that lack adverse side effects.

One potential new approach to treat AKs/SCCIS would be to test the efficacy of topical small-molecule kinase inhibitors (SMKIs) targeting drivers of growth in lesional keratinocytes.8
One biologically plausible class of kinases to target is the Src-family tyrosine kinases (SFKs), which are known drivers of human cSCCs and shown to be activated in human AKs/SCCIs. The other key consideration is to identify molecules that can effectively penetrate the thickened stratum corneum and epithelial layers found in AKs/SCCIs/cSCCs. It has been shown that the human epidermal barrier may be permeable to some organic molecules with a size of 500 daltons or less. We identified dasatinib, a small-molecule inhibitor of SFKs with a molecular weight of 488 daltons, as a potential candidate molecule that may penetrate the epidermal barrier to significantly inhibit SFKs driving AK/SCCIs growth and induce lesion regression.

2 | QUESTIONS ADDRESSED

We hypothesized that topical kinase inhibitors targeting tyrosine kinases or downstream oncogenic pathways might be effective for treating skin cancer. To test this hypothesis, we used K14-Fyn Y528F transgenic mice which represent an in vivo skin cancer model that produces cSCCs and precancerous lesions analogous to corresponding human lesions. In this model, the cSCCs show activation of SFKs and downstream oncogenic signalling pathways, including the PI3K/mTOR, Ras/MAPK and JAK/STAT pathways. This transgenic model provides a useful tool for screening topical kinase inhibitors that may be efficacious in treating skin cancer.

Using this model as a screening tool, we applied topical dasatinib and BEZ235, an inhibitor of the PI3K/mTOR pathway, to cSCCs and compared their efficacy to induce cSCC regression against vehicle and the first-line topical agent, 5-fluorouracil.

3 | EXPERIMENTAL DESIGN

Five- to six-week-old K14-Fyn Y528F mice with cSCCs were divided into 3 cohorts for topical treatment: control (18 cSCCs), dasatinib (25 cSCCs) and 5-FU (18 cSCCs). Inclusion criteria were that cSCCs needed to be 3 x 3 mm or larger; such lesions have keratotic scale and a thickness similar or greater than that seen in human AKs/SCCIs which is a more stringent therapeutic test than treating smaller lesions that occur in this model. Prior to treatment, cSCCs were measured with callipers in the two longest dimensions to calculate tumor area and then photographed. These procedures were repeated weekly during treatment to follow cSCC size for treatment efficacy. cSCCs were treated daily (7x/week) with vehicle (DMSO), dasatinib solution (0.89%) and 5% 5-FU. In analogous experiments, mice with cSCCs were divided into 2 cohorts for topical treatments: vehicle control (10 cSCCs) and BEZ-235 (1%) (15 cSCCs). Biopsies of representative cSCCs were taken at time 0 and at the indicated time points to assess tumor regression, inflammation and epidermal ulceration.

4 | METHODS

4.1 | In vivo cSCC model

The K14-Fyn Y528F transgenic mouse was developed and characterized as previously described and is available from Jackson Laboratories: FVB.Cg-Tg(KRT14-Fyn*)aJsey/J. This transgenic mouse spontaneously develops cSCCs that resemble human lesions at the histologic and molecular levels. Study inclusion criteria for mice: (a) genotype positive, (b) non-breeding and (c) cSCCs greater than 3 mm in diameter were included in the study. The cohorts of mice were as follows: Veh (DMSO)-8 mice with 18 cSCCs, average of 2.3 tumors per mouse; dasatinib-15 mice with 25 cSCCs, average of 1.7 tumors per mouse; 5-FU-8 mice with 13 cSCCs, average of 1.6 tumors per mice; Veh (DMF)-8 mice with 10 cSCCs, average of 1.3 cSCCs per mouse; BEZ-235-4 mice with 15 tumors, average of 3.8 cSCCs per mouse. The five cohorts of mice control Veh (DMSO), 5-FU, dasatinib, Veh (DMF) and BEZ235 were matched for age and closely for sex.

4.2 | Topical application of dasatinib, BEZ235 and 5-FU

DMSO was used as the vehicle control. Dasatinib (LC Laboratories #D-3307) was dissolved in DMSO at a concentration of 20 mmol/L equivalent to 0.89%. BEZ-235 (LC Labs Cat. No. N-4288) was dissolved in dimethylformamide (Fisher Cat No. AA43997AE) at 1% (g/g). Topical 5-fluorouracil (5-FU, 5% cream, Taro Pharmaceutical Industries, Ltd. NDC 51672-4118-6). Mice received approximately 100-150 mg of cream topically per day on each cSCC which contains 5-7.5 mg of 5-FU. All topical agents and controls were applied once daily to cSCCs with a cotton swab to uniformly cover the surface of the lesion.

4.3 | Monitoring cSCC size during treatment

cSCCs were photographed with a ruler at time 0 and weekly during topical treatment. The area of each cSCC was determined using callipers to measure two longest orthogonal dimensions at time 0 and weekly during treatment. Tumor measurements were reported as mm² and ceased if skin became ulcerated or the murine host died. Relative tumor area as a function of time was calculated by using the initial tumor area as 100%, and subsequent tumor areas were compared to time 0 area.

4.4 | Tissue histology and immunohistochemistry

cSCC tumor tissue was harvested from representative lesion prior to initiating treatment, at 2-3 weeks and at the end of treatment. Harvested tissues were fixed in 10% formalin for tissue processing. Histologic slides with haematoxylin and eosin staining were reviewed. The neutrophilic inflammatory response was assessed by morphology on H&E staining, inflammatory cells with...
a segmented nucleus and cytoplasmic granules. Murine CD3-positive T cells were detected using immunohistochemistry with a CD3 antibody, 1:100 (Abcam, Cambridge, MA). To assess cell numbers, five square millimetres of dermis was examined to determine the number of T cells and neutrophils in each biopsy specimen. Cells in the epidermis, hair follicle or fat layer were not counted. Biopsies from three different mice in the indicated cohorts were evaluated. Statistical significance was determined using Student’s t test for the means.

Tissue sections were subjected to immunohistochemistry staining as previously reported (Zhao, 2009). Phosphorylated Src kinases were detected using pY416 rabbit Ab from Cell Signaling, #2101 at a 1:20 dilution. Total Src kinases were detected using the SRC2 (sc-18) antibody from Santa Cruz at a 1:100 dilution. All photomicrographs were obtained using a Keyence microscope.

4.5 Statistical analysis

All statistical calculations were performed using Student’s t test for comparison of the means.

5 RESULTS

Topical dasatinib (0.89%) applied daily induced 45% and 77% regression of cSCCs after 2 and 5 weeks of treatment, respectively, compared to control, p < 0.05 (Figure 1A, B, C, D and F). Topical 5-FU (5%) induced 70% regression at 2 weeks. cSCC regression with 5-FU was associated with epidermal ulceration in 2/15 of tumors, and 7/8 mice died by day 18 of 5-FU treatments (Figure 1A, D and G). No cSCC ulceration or murine mortality was seen in the dasatinib or control cohorts (Figure 1G). During the first 3 weeks of vehicle application, control cSCCs enlarged. After 5 weeks of vehicle application, the control cSCCs only lost 12% of relative tumor area.

Immunohistochemical analysis of representative lesions from control and dasatinib-treated cSCCs demonstrated lower levels of activated Src kinases compared to vehicle-treated and untreated lesions (Figure 1E). Immunohistochemical analysis for total Src kinases showed a staining pattern similar to activated Src kinase in all three sets of lesions (Figure 1E). However, staining for total Src kinase demonstrated broader staining but much weaker in the dasatinib-treated lesions (Figure 1E). These data indicate that dasatinib treatment may activate cellular mechanisms that downregulate activated Src kinases. The data show that dasatinib-induced regression of the cSCCs is associated with decreased levels of activated and total Src kinases that approximate levels seen in unremarkable epidermis.

The level of inflammation associated with topical dasatinib and 5-FU treatment was determined by assessing the number of dermal CD3-positive T cells and neutrophils in three representative treated lesions (Figure 1E). Immunohistochemical analysis for total Src kinases showed a staining pattern similar to activated Src kinase in all three sets of lesions (Figure 1E). However, staining for total Src kinase demonstrated broader staining but much weaker in the dasatinib-treated lesions (Figure 1E). These data indicate that dasatinib treatment may activate cellular mechanisms that downregulate activated Src kinases. The data show that dasatinib-induced regression of the cSCCs is associated with decreased levels of activated and total Src kinases that approximate levels seen in unremarkable epidermis.

The level of inflammation associated with topical dasatinib and 5-FU treatment was determined by assessing the number of dermal CD3-positive T cells and neutrophils in three representative treated lesions from each cohort. The level of inflammation associated with treatment was determined by counting CD3-positive T cells and neutrophils in the dermis at the treatment site. Dasatinib treatment of cSCCs did not increase levels of CD3-positive T cells or neutrophils compared to untreated cSCCs and vehicle-treated lesions (Figure 2). In contrast, topical 5-FU induced inflammation and was associated with higher numbers of CD3-positive T cells (P = 0.03) and neutrophils (P = 0.04) compared to dasatinib-treated mice. In some cases, topical 5-FU induced epidermal ulceration (Figures 1 and 2). Overall, dasatinib induced cSCC regression similar to topical 5-FU with less inflammation and no ulceration.
Since SFKs are known to activate the PI3K/mTOR pathway in the K14-Fyn Y528F model, we determined whether topical application of BEZ-235, a small-molecule PI3K/mTOR inhibitor, to cSCCs induced regression similar to that seen with dasatinib. Topical application of 1% BEZ-235 induces regression of cSCCs by over 60% compared to vehicle-treated lesions by 5 weeks without significant erythema or ulceration (Figure S1). The BEZ-235-induced regression was found to be statistically significant at each weekly time point P < 0.01 (Figure S1).

**CONCLUSIONS**

As a first step in identifying new approaches to treat skin cancer, we hypothesized that topical application of small-molecule kinase inhibitors could inhibit the growth of cSCCs in the K14-Fyn Y528F skin cancer model. It has been demonstrated that human AKs, SCCIS and cSCCs contain increased levels of activated Src kinases compared with adjacent unremarkable epidermis. These data support the hypothesis that Src kinases represent a potential therapeutic target in human AKs/SCCIS/cSCCs.

To further test this hypothesis, cSCCs were treated once daily with 0.89% topical dasatinib; treated cSCCs demonstrated almost 80% regression by 5 weeks while vehicle-treated lesions did not demonstrate significant regression. cSCCs treated with dasatinib demonstrated a similar level of cSCC regression comparable to topical 5% 5-FU after 2 weeks of daily application. However, topical dasatinib induced cSCC regression without inducing prominent inflammation or ulceration as was seen with 5-FU. In addition, dasatinib was well tolerated by mice while topical 5-FU led to mortality after 2 weeks. The mortality rate noted in the K14-Fyn Y528F mice treated with topical 5-FU is elevated compared to other studies. This may be due to increased drug absorption due to impaired skin barrier formation in the cSCCs leading to elevated systemic levels or to increased production of toxic metabolites in the K14-Fyn Y528F line.

In parallel experiments, topical BEZ-235 also induced regression of cSCCs compared to vehicle without inducing ulceration or significant erythema. Together, these data suggest that topical application of small-molecule kinase inhibitors targeting key kinases driving keratinocyte growth may be useful for treating cSCCs and related precursor lesions. These data also raise the possibility that topical application of multiple small-molecule kinase inhibitors together targeting the drivers of AKs/SCCIS/cSCCs may be more effective than a single compound.

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**CONFLICTS OF INTEREST**

JTS and the University of Pennsylvania are co-owners of US patents 9 084 790 B2 and 9 555 034 B2 outlining the use of topical small-molecule kinase inhibitors to treat skin cancer. These patents have not yet been subject to a licensing agreement.

**AUTHOR CONTRIBUTIONS**

XY, AD, SS, JH, JB, CM, TD, FS, SD and JH performed experiments described in this paper. XY, AD, SS, JB, TD, VL, HS, AF and JS evaluated data and constructed figures. HS, AF and JS provided facilities and financial support for this research. XY, AD, VL, HS, AF and JS wrote and edited the manuscript.

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**REFERENCES**


SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Topical BEZ-235 induces cSCC regression without ulceration.