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Dual Inhibition of Epidermal Growth Factor Receptor and Insulin-like Growth Factor Receptor I Reduces Angiogenesis and Tumor Growth in Cutaneous Squamous Cell Carcinoma

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Abstract

Purpose—Cutaneous squamous cell carcinoma (CSCC) is the second most common non-melanoma skin cancer. The majority of the ~250,000 cases occurring annually in the United States are small, non-aggressive, and cured by excision alone. However, a subset of these tumors which are defined by poorly differentiated histology, large tumor size, invasion of adjacent structures and/or regional metastases can prove resistant to treatment despite adjuvant radiotherapy and have increased risk of recurrence and nodal metastasis. Novel therapeutic approaches are necessary to improve outcomes for patients with aggressive CSCC.

Experimental Design—We analyzed the effect of targeted therapy on the growth and survival of CSCC cell lines using an anti-IGF-IR antibody, A12, alone or in combination with an anti-EGF-R antibody, cetuximab, both *in vitro* and *in vivo* in an athymic nude mouse model of CSCC.

Results—Treatment with A12 and cetuximab inhibited the signaling pathways of IGF-IR and EGFR and inhibited proliferation and induced apoptosis of SCC cell lines *in vitro*. Immunohistochemical staining revealed decreased proliferating cell nuclear antigen (PCNA) and microvessel density (MVD) as well as increased apoptosis within the treated tumor xenografts. In addition, the administration of A12, alone or in combination with cetuximab inhibited the growth of tumors by 51% and 92% respectively, and significantly enhanced survival in the nude mouse model of CSCC ($p = 0.044$ and $p < 0.001$ respectively).

Conclusions—These data suggest that dual treatment with monoclonal antibodies to the EGFR and IGF-IR may be therapeutically useful in the treatment of CSCC.

Keywords

Insulin-like growth factor receptor I (IGF-IR); epidermal growth factor receptor (EGFR); squamous cell carcinoma; orthotopic model; preclinical study

Introduction

Cutaneous squamous cell carcinoma (CSCC) is the second most common type of non-melanoma skin cancer. The annual incidence is estimated at 1 case per 1000 individuals and approximately 250,000 cases of CSCC occurred in 2001 alone¹. The cause-specific mortality rate due to CSCC is less than 1% overall, but rises to 30% at 3 years for those patients whose tumors have aggressive features². CSCC incidence has risen dramatically over the past 2 decades because of lifestyle changes leading to increased voluntary exposure to sunlight³. The standard of care for the management of CSCC is surgical excision. However, even adequate initial treatment of poorly differentiated CSCC can be complicated by a recurrence rate of up to 25%. Recent successes involving the therapeutic use of antibodies and small molecule inhibitors against tyrosine kinases have generated considerable interest in research aimed at targeting these receptors in a wide variety of malignancies. In an attempt to improve the treatment of CSCC, we explored the effect of inhibition of two of these receptors on cutaneous tumor growth *in vitro* and *in vivo*.

The insulin-like growth factor I receptor (IGF-IR) is a ubiquitous transmembrane tyrosine kinase composed of two extracellular alpha subunits and two intracellular beta subunits^{4–6}. Ligand binding (IGF1 or IGF2) to the extracellular alpha subunits triggers conformational changes in the beta subunits activating the receptors tyrosine kinase activity, which in turn activates downstream signaling cascades, including the phosphatidylinositol 3-kinase/AKT and Ras/Raf/mitogen-activated protein kinase (MAPK) pathways^{6–11}. Numerous human tumors have been shown to overexpress IGF-IR or have increased IGF-IR kinase activity resulting in enhanced proliferation, protection from apoptosis, stimulation of migration and invasion, and stimulation of angiogenesis. Targeted therapies, including insulin-like growth factor (IGF) binding proteins, human monoclonal antibodies and small-molecule tyrosine kinase inhibitors against IGF-IR, have been developed and show promise for therapeutic use in both *in vitro* and *in vivo* experiments^{12–16}. A12, a high-affinity human monoclonal antibody to IGF-IR, has been shown to induce apoptosis and inhibit tumor growth by competitively binding to the receptor and inducing IGF-IR internalization and downregulation. Experimentally, A12 has been shown to inhibit the growth of breast, pancreatic, colon, and renal tumors, both *in vitro* and *in vivo* with little toxicity or weight loss in nude mouse models¹³.

The epidermal growth factor receptor (EGFR), a member of the ErbB tyrosine kinase receptor family, is a transmembrane glycoprotein receptor. Activation of EGFR stimulates phosphorylation of downstream signaling cascades that ultimately regulate cell proliferation, migration, adhesion, differentiation, and survival^{17–19}. EGFR is frequently overexpressed in mucosal squamous cell carcinoma and is associated with malignant phenotype and poor prognosis^{20, 21}. Less is known about the expression of EGFR in cutaneous squamous cell carcinoma. Several small studies have shown that 43–80% of CSCCs express membranous EGFR but this increases to 100% for metastatic CSCC. In primary tumors, Fogarty et al. demonstrated baseline activation of EGFR in 5/9 specimens with detectable EGFR expression. While cetuximab has been well-studied for the treatment of mucosal squamous cell carcinomas, the benefit for CSCC is not well understood.^{22–25}. Barnes et al. have shown *in vitro* efficacy of an EGFR inhibitor, Iressa on CSCC and several case reports have

examined the efficacy of various EGFR inhibitors and have suggested the benefit of combination therapy with a second agent.

EGFR and IGF-IR are logical targets for molecular therapy of cancer based on their frequent overexpression and established roles in the pathogenesis and progression of numerous cancers^{18, 19, 26}. Recently, dual inhibition of receptor tyrosine kinases has emerged as a method to improve the efficacy of targeted therapy. Previous studies of single agents have shown that tumors often have complex regulation involving multiple protein tyrosine kinases and may use these pathways as escape mechanisms when a single receptor is targeted^{25, 27}.

In this study, we analyzed the effects of targeted therapy against IGF-IR and EGF-R on CSCC cell lines. We hypothesize that targeted therapy against IGF-IR (A12) and EGFR (cetuximab) will inhibit CSCC tumor growth *in vitro* and in an athymic nude mouse model.

Materials and Methods

Cell Lines and Culture Conditions

The CSCC cell lines Colo16, SRB1, and SRB12 were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), sodium pyruvate, L-glutamine, vitamins, non-essential amino acids (all from Life Technologies, Rockville, MD), and penicillin-streptomycin (Flow Laboratories, Rockville, MD). Adherent monolayer cultures were maintained on plastic and incubated at 37° C in an atmosphere of 5% carbon dioxide and 95% air. The cultures were maintained no longer than 12 weeks after recovery from frozen stocks. These 3 cell lines were genotyped using short tandem repeat analysis and have been found to be unique and distinct from other cell lines in the American Type Culture Collection and our laboratory.

Animals and Maintenance

Male athymic nude mice, age 8 to 12 weeks, were purchased from the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD). The mice were housed and maintained in laminar flow cabinets under specific pathogen-free conditions in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care. The mice were used in accordance with the Animal Care and Use Guidelines of The University of Texas M.D. Anderson Cancer Center (Houston, TX) under a protocol approved by the Institutional Animal Care and Use Committee.

Reagents

Cetuximab (ImClone Systems Incorporated, Branchburg, NJ) was diluted in phosphate-buffered saline (PBS) to the appropriate concentrations for *in vitro* studies and at a concentration of 5 mg/ml for intraperitoneal injections in the animal study. A12 was generously provided by ImClone Systems Incorporated. For *in vitro* administration, A12 was dissolved in PBS to a concentration of 10 mg/ml and further diluted to appropriate final concentration in RPMI 1640 medium with or without 2% FBS as described below. For *in vivo* testing, A12 was dissolved in PBS to a concentration of 4 mg/ml. Both cetuximab and A12 solutions were prepared immediately before administration to the mice.

The following antibodies were used: anti-IGF-IR β (C-20) and anti-EGFR (Santa Cruz Biotechnology, Santa Cruz, CA); anti-phosphorylated IGF-IR (Tyr 1131)/IR (Tyr 1146), anti-phosphorylated EGFR (Tyr1068), anti-AKT, anti-phosphorylated AKT (Ser473), anti-mitogen-activated protein kinase (MAPK) mouse (p42), anti-phosphorylated MAPK (Tyr42/44) (Cell Signaling Technology, Beverly, MA); anti- β -actin (Sigma, St. Louis, MO);

mouse anti-proliferating cell nuclear antigen (PCNA) clone PC-10 (DAKO A/S, Copenhagen, Denmark); rat anti-mouse CD31/platelet-endothelial cell adhesion molecule-1 and rat anti-mouse CD31 peroxidase-conjugated rat anti-mouse IgG1 (PharMingen, San Diego, CA); peroxidase-conjugated goat anti-rabbit IgG and peroxidase-conjugated goat anti-rat IgG1 (Jackson ImmunoResearch Laboratories, West Grove, PA); peroxidase-conjugated rat anti-mouse IgG2a (Serotec, Harlan Bioproducts for Science, Inc., Indianapolis, IN); Hoechst Dye 3342 MW 615.9 (Hoechst, Warrington, PA), and Alexa Fluor 594-conjugated goat anti-rat IgG and Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR). The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was done using a commercial apoptosis detection kit (Promega, Madison, WI).

Western Blotting

To measure EGFR and IGF-IR expression and phosphorylation in CSCC lines, total cell protein extracts were obtained from Colo16, SRB1, and SRB12 cells. The cells were grown to sub-confluence in DMEM supplemented with 10% FBS, washed with PBS, and scraped with lysis buffer as previously described²⁸. The proteins (50 µg) were resolved by polyacrylamide gel electrophoresis and electrophoretically transferred onto nitrocellulose membranes. The membranes were blocked with 1% bovine serum albumin (BSA) (Sigma-Aldrich) in 0.1% Tween 20 (v/v) in Tris-buffered saline for 30 minutes, then probed overnight with desired primary antibodies. After incubation with the secondary antibody, signals were visualized using enhanced chemiluminescence (Pierce, Rockford, IL).

We performed Western blotting to demonstrate that cetuximab and A12 are able to specifically inhibit the phosphorylation EGFR and IGF-IR *in vitro*. Western immunoblotting was performed. Cells from all three lines were incubated in serum-free medium for 24 hours. Then, cells were incubated with either A12 (50nM), cetuximab (1 µg/ml) or combination of A12 and cetuximab for 2 hours. Untreated cells were used as a control. The cells were then stimulated with both IGF-I (10nM) (R&D Systems, Minneapolis, MN) and EGF (10nM) (Upstate biotechnology, Lake Placid, NY) for 15 min. Cells were then processed and western blotting analysis performed as described above.

Colo16 cells were further used to test the ability of cetuximab and A12 to inhibit the EGFR and IGF-IR signaling pathways. After serum starvation overnight, the cells were incubated with no cetuximab, A12, or the combination; untreated cells were used as control. The cells were stimulated with both IGF-I and EGF for 15 minutes. Cells were then processed and Western blot analysis performed as described above.

Measurement of Cell Proliferation

We used a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay to test the ability of cetuximab and A12 to inhibit the proliferation of all three CSCC lines *in vitro*. Two thousand cells per well were grown in DMEM medium supplemented with 10% FBS in 96-well tissue culture plates. After 24 hours, the cells were treated with various concentrations of A12 (0.1 – 100nM) or cetuximab (0.01–50ug/ml) diluted in DMEM medium supplemented with 2% FBS. To measure the number of metabolically active cells after a 3-day incubation period, we used an MTT and quantified the results at an optical density of 570 nm using a 96-well microtiter plate reader (MR-5000; Dynatech Laboratories, Chantilly, VA).

Measurement of Cell Death

Colo16 cells were plated at a density of 2×10^5 cells per well in 38-mm² six-well plates (Costar, Cambridge, MA) and maintained for 24 hours. Then, A12 was added in various

concentrations (0 – 100nM) with or without cetuximab (5µg/ml) in DMEM medium supplemented with 2% FBS. After 48 hours, the extent of cell death was determined by propidium iodide (PI) staining of hypodiploid DNA. For PI staining, the treated cells were resuspended in a Nicoletti buffer (50µg/ml PI (Sigma-Aldrich), 0.1% sodium citrate and 0.1% Triton X-100) for 20 minutes at 4°C. Cell cycle data were then analyzed by flow cytometry, and the sub-G0/G1 fraction was measured using the Multicycle System (Phoenix Flow System, San Diego, CA).

Effects of A12 and Cetuximab on the Growth of Cutaneous Squamous Cell Carcinoma Xenografts in Nude Mice

CSCC xenografts in nude mice were established as described previously^{28, 29}. Briefly, 1×10^6 cells in an injection volume of 100µl were injected subcutaneously into the right side of the face of each mouse using a 30-gauge needle. The tumors were allowed to develop for 7 days. The mice were then randomized into four groups (10 mice per group), and the drugs were administered as follows: 1) A12, 40 mg/kg twice per week by intraperitoneal (IP) injection, 2) cetuximab, 50 mg/kg twice per week IP, 3) both A12, 40 mg/kg, and cetuximab, 50 mg/kg, IP, twice per week, 4) 250µl of PBS administered intraperitoneally twice per week as placebo.

The mice were treated for 18 days and the weighed twice per week. Animals would have been euthanized if they lost >20% of body weight or if they became moribund. However, all the animals maintained their weights and none required sacrifice due to the above criteria prior to the end of the treatment period. At the end of the 18 days treatment period, the mice were euthanized by CO₂ asphyxiation and necropsy was performed. The cervical lymph nodes, the lungs, and the skin tumors were removed, sectioned, stained with hematoxylin-eosin (H&E), and examined for the presence of metastasis. During necropsy, the tumor size was measured in two dimensions and the volume was determined using the formula $V = (\text{length}) (\text{width}^2) \pi/6$, expressed in cubic millimeters. Drug treatment continued until the day of euthanasia with the last dose administered two hours prior to euthanasia. Tumor inhibition was calculated as a percentage according to the formula $[1 - (T/C)] \times 100$ where T and C represent the mean tumor volumes of the treatment group and the control group, respectively.

For immunohistochemical and routine H&E staining, half of the tumor was fixed in formalin and embedded in paraffin. The remainder was embedded in OCT compound (Miles Inc., Elkhart, IN), rapidly frozen in liquid nitrogen, and stored at -80°C.

Effects of A12 and Cetuximab on the Survival of Nude Mice Bearing Cutaneous Squamous Cell Carcinoma Xenografts

CSCC xenografts were established in nude mice as described above. Seven days after the tumor cell injection, the mice were randomized into four groups (five mice per group): control, A12, cetuximab, and combination group. Each group of mice was treated with PBS, A12, cetuximab, or both agents as described in the previous section. The mice were weighed twice per week and euthanized if the animals showed weight loss of >20% or appeared moribund. The mice were treated for 5 weeks.

Immunohistochemistry on Murine Tumor Tissue Sections

For IGF-IRβ, EGFR and PCNA staining, paraffin-embedded sections were first de-paraffinized in xylene. Excess xylene was removed by washing the slides in ethanol. The tissue was treated with pepsin for 20 min at 37°C. Immunohistochemistry was performed as previously described³⁰.

For staining with antibodies against pIGF-IR β , pEGFR, and CD31/PECAM-1, frozen tumors were sectioned (8 to 10 mm thick), mounted on positively charged Superfrost slides (Fisher Scientific, Pittsburgh, PA), air dried for 30 minutes, and fixed in cold acetone for 10 minutes). Immunohistochemistry was performed as previously described³⁰.

For TUNEL staining: tissues were fixed with 4% paraformaldehyde (methanol free) for 10 min at room temperature, washed twice with PBS for 5 minutes, and then incubated with 0.2% Triton X-100 for 15 minutes at room temperature. Then the tissue sections were incubated with reaction buffer containing 44 μ L of equilibration buffer, 5 μ L of nucleotide mix, and 1 μ L of terminal deoxynucleotidyl transferase (Promega kit) at 37°C for 1 hour, avoiding exposure to light. The reaction was terminated by immersing the samples in 2X saline-sodium citrate buffer for 15 minutes. The samples were then washed three times for 5 minutes with PBS to remove unincorporated fluorescein-dUTP.

Immunofluorescence microscopy was performed using a Zeiss Axioplan2 microscope (Carl Zeiss, Thornwood, NY.) equipped with a 100-Watt HBO mercury bulb and filter sets (Chroma Inc, Brattleboro, VT) to individually capture red and blue fluorescent images. Images were captured using a C5810 Hamamatsu color chilled 3-chip charge-coupled device camera (Hamamatsu, Hamamatsu City, Japan) and digitized using Optimas imaging software (Silver Spring, MD). The stained sections were imaged with a Microphot-FX microscope (Nikon, Melville, NY) equipped with a three-chip-charged couple device (CCD) color video camera (Model DXC990; Sony Corp, Tokyo, Japan).

For evaluation of PCNA and CD31 staining, the mean positive area was quantified in five random 0.159 mm² fields (magnification, $\times 100$) per slide from five slides per study group using Image-Pro Plus software package (Media Cybernetics, Inc., Silver Spring, MD). For TUNEL staining quantification, the labeled cells were counted in five random 0.159 mm² fields (Magnification, $\times 100$) per slide from total of five slides per study group. The photomontages were prepared using Photoshop software (Adobe Systems, San Jose, CA).

Statistical Analysis

The non-parametric Wilcoxon rank-sum test was used to assess differences in mouse tumor volume between each treatment group and the control group. We ran a repeated-measures regression model with treatment, time, and treatment by time interaction to compare the tumor volumes observed for the group receiving A12 plus cetuximab with the group receiving cetuximab alone. This methodology was also used to compare tumor volumes from the group receiving A12 plus cetuximab to the group receiving A12 alone. Survival was analyzed with the Kaplan-Meier method. Differences between treatment and control groups were compared with the log-rank test. We compared the results from PCNA, CD31 and TUNEL staining by independent-samples t-test. Least-squares fit curves were generated for the MTT and PI assays. SPSS 12.0 for Windows software (SPSS Inc., Chicago, IL) was used for all statistical analysis. A p-value of 0.05 was considered significant.

Results

EGFR and IGF-IR are Expressed by Cutaneous Squamous Cell Carcinoma Cell Lines

Western blot analysis revealed that both cell lines tested stained positively for EGFR and IGF-IR (Figure 1). Only the Colo16 cell line exhibited baseline phosphorylation of both EGFR and IGF-IR; the SRB12 cell line showed weak positive for pIGF-IR.

A12 and Cetuximab Treatment Leads To Decreased Phosphorylation Of Their Target Receptors In Cutaneous Squamous Cell Carcinoma Cell Lines

We evaluated the ability of A12 and cetuximab to inhibit the phosphorylation of EGFR and IGF-1R in the Colo16 and SRB1 cell lines (Figure 1). Under basal conditions in serum-free medium, there was a low level of EGFR autophosphorylation, which was enhanced after exposure to EGF (Figure 1). Baseline expression of pIGF-1R was minimal; however, phosphorylation occurred upon stimulation with IGF-1. In contrast, there were low levels of pIGF-1R but not pEGFR at baseline in SRB1 cells; both receptors were similarly activated with growth factor stimulation (Figure 1B). Treatment with A12 blocked phosphorylation of IGF-1R and resulted in decreased expression of IGF-1R in both cell lines, but this effect was more pronounced for Colo16. EGFR phosphorylation was blocked by cetuximab treatment but not impacted by A12 treatment in both cell lines. No differences in total EGFR expression levels were noted in either cell line.

Treatment with A12 and Cetuximab Decreases Proliferation of Certain Cutaneous Squamous Cell Carcinoma Cell Lines

We examined effect of A12 treatment on the proliferation of two CSCC cell lines (Colo16 and SRB1) using the MTT assay. The growth of cells incubated with increasing concentrations (0–100nM) of A12 in DMEM containing 2% serum was determined 72 hours after addition of drug (Figure 2A). Treatment with various concentrations of A12 did not significantly inhibit proliferation in any of the cell lines studied, although a trend toward inhibition of the Colo16 cells was noted. Next, we performed MTT assays using the Colo16 cell line to compare the effect of treatment with cetuximab with the A12/cetuximab combination on cell proliferation. Proliferation of Colo16 cells was inhibited by treatment with cetuximab alone, and to a greater extent by the combination of A12 and cetuximab in a dose-dependent manner ($p < 0.01$, Figure 2B).

Treatment with A12 and Cetuximab Leads to Induction of Apoptosis in the Colo16 Cell Line

We examined the effect of dual inhibition of EGFR and IGF-1R on the rate of apoptosis of the Colo16 cell line using PI and flow cytometry. Treatment with A12 alone at increasing concentration did not significantly alter the baseline rate of apoptosis, whereas combination treatment with cetuximab lead to induction apoptosis (11.9% vs. 22.7%, $p < 0.012$)(Figure 3).

Treatment with A12, Cetuximab, or an A12/Cetuximab Combination Inhibits Tumor Growth in a Nude Mouse Model of Cutaneous Squamous Cell Carcinoma

The Colo16 cell lines was used for all *in vivo* experiments based on the strength of inhibition of intracellular signaling by A12 and cetuximab that was noted previously (Figure 1). Cells were injected into mice as described with visually evident tumors noted at 1 week after inoculation. Treatment with either A12 or cetuximab alone reduced the growth of Colo16-derived SCC xenografts in nude mice by 51% ($p = 0.058$) and 49% ($p = 0.041$), respectively when compared with the control group (Fig. 4A). Mice treated with A12 in addition to cetuximab showed a 92% decrease in the mean estimated tumor volume when compared with the control group ($p < 0.001$) or animals treated with either cetuximab or A12 alone ($p < 0.05$).

Treatment with A12 Alone and in Combination with Cetuximab Improves Survival in an Orthotopic Nude Mouse Model of Cutaneous Squamous Cell Carcinoma

A12 and cetuximab were both well tolerated by the animals without substantial adverse effects. None of the animals required sacrifice due to weight loss or systemic malignancy,

but rather were euthanized when the local tumor burden became excessive (defined as > 1 cm in the maximum dimension or significant ulceration). Treatment with A12 or cetuximab alone resulted in significantly greater survival as compared to the control group ($p = 0.044$ and $p = 0.023$, respectively) (Figure 4B). The combination treatment group also demonstrated a significantly improved survival rate as compared with the control ($p < 0.001$) or A12 only ($p < 0.01$) groups. There was no statistically significant difference in survival between the combination treatment group and the group treated with cetuximab alone ($p > 0.1$).

Treatment of Cutaneous Squamous Cell Carcinoma Cell Lines with A12, Cetuximab, and A12/Cetuximab Combination in an Orthotopic Tumor Model Leads to Induction of Apoptosis, Decreased Cell Proliferation, and Inhibition of Angiogenesis

The effect of A12 and cetuximab on cellular proliferation was determined by assessing the expression of PCNA in tumor sections harvested at necropsy from mice in all treatment groups. Mean positive area analysis of PCNA staining showed a significant decrease in cell proliferation in mice treated with either cetuximab ($p = 0.016$) or A12 alone ($p = 0.026$) as compared with the control group (Figure 5A and 5B). Moreover, there was an additional decrease in cell proliferation in mice treated with A12/cetuximab combination as compared with the control group ($p < 0.001$). However, the effects of combination treatment on cell proliferation were not significantly different from treatment with either A12 or cetuximab alone ($p > 0.1$).

To assess the degree of intratumoral apoptosis, the tumor sections were stained for DNA fragmentation using the TUNEL method (Figures 5A and 5C). The mean number of apoptotic cells per unit area ($\pm$ SD) in the tumors of control mice and the mice treated with cetuximab were 11.8 ± 7.4 and 18.2 ± 9.0 , respectively ($p = 0.102$). Treatment with either A12 alone or A12 in combination with cetuximab increased the mean number of apoptotic cells per unit area to 39.7 ± 20.3 and 35.3 ± 13.6 respectively, representing a significant increase in intratumoral apoptosis when compared with the control group ($p = 0.001$ and $p = 0.001$) or cetuximab only group ($p = 0.007$ and $p = 0.016$).

To determine the intratumoral microvessel density, we stained tumor sections with CD31-specific antibodies. Treatment with either A12 or cetuximab alone resulted in a statistically significant inhibition of tumor-associated angiogenesis as defined by the mean CD 31 positive area when compared with the control group ($p < 0.001$ and $p < 0.001$, respectively; Figures 5A and 5D). Treatment with A12 in combination with cetuximab resulted in an additional inhibition of tumor associated angiogenesis when compared with the control group ($p < 0.001$) or the group treated with cetuximab only ($p = 0.003$).

Discussion

Our findings indicate that the combination of A12 and cetuximab simultaneously block EGFR and IGF-IR activation and significantly reduce tumor volume by both direct antitumor and angiogenic effects. We showed that elevated IGF-IR and EGFR expression is consistently and concurrently elevated in CSCC cell lines. In an orthotopic nude mouse model of CSCC, dual inhibition with A12 and cetuximab reduced tumor volume by 92% as compared with approximately 50% with either agent alone. Combination treatment also significantly improved survival. In vitro studies demonstrated the inhibition of activation of IGF-IR by A12 and EGFR by cetuximab, but showed no cross-inhibition. The combination of A12 and cetuximab also showed direct growth inhibitory and apoptotic activity against CSCC cell lines. In vivo, monotherapy with either A12 or cetuximab caused a significant increase in apoptosis and decrease in both cellular proliferation and microvessel density as compared to control, an effect which was enhanced for combination treatment.

A12 treatment resulted in downregulation of the total IGF-IR expression levels as has been demonstrated by others¹⁸. These results are also consistent with previous studies of EGFR and IGF-IR overexpression in CSCC specimens and cell lines^{5, 31}. We also showed that A12 has effects on downstream kinases, such as AKT, confirming similar findings by Wang et al³².

Both A12 and cetuximab when used as single agents demonstrated only minor antiproliferative activity on CSCC cell lines, while combination treatment led to a significant inhibition of growth ($p < 0.01$). This finding is consistent with multiple studies demonstrating the efficacy of dual inhibition of EGFR and IGF-IR pathways in inhibiting tumor cell proliferation as compared to targeting a single pathway³¹. In contrast to studies of small molecule IGF-IR kinase inhibitors, treatment with A12 alone did not significantly increase apoptosis of CSCC *in vitro*. These data suggest that A12 exerts its anti-proliferative effect mainly through a cytostatic, rather than cytotoxic mechanism. Similar results have been reported in other studies using single agent inhibitors including IGF-IR antibodies (EM164, h7C10, and CP-751,871) and inhibitors of EGFR including AG 1478, mAb225, and gefitinib^{15, 28, 29, 33, 34}.

Treatment with A12 and cetuximab significantly inhibited SCC tumor growth in the murine model. Either agent alone resulted in approximately a 50% reduction in tumor volume while treatment with a combination of the two drugs resulted in a greater than 90% reduction in tumor volume. Similarly, longer survival was also found for the combined treatment group as compared with control or A12 treatment groups. Differences in sensitivity to inhibition of either EGFR or IGF-IR alone have previously been found in other tumors³⁵. However, the concurrent expression of both IGF-IR and EGFR together in CSCC appears to be important for tumor growth and development, and simultaneous inhibition of these two tyrosine kinases results in a significantly greater reduction of tumor development and growth. Our findings are consistent with other studies suggesting that targeted therapy of IGF-IR can be used in combination with other therapeutic strategies in order to achieve maximum anti-tumor effects^{26, 27, 36} and that combination therapy may be beneficial in preventing the development of drug resistance, such as that seen with trastuzumab (Herceptin), erlotinib, and gefitinib^{27, 37, 38}.

Immunohistochemical analyses of tumor sections from mice treated with either A12 or cetuximab alone revealed a significant decrease in the proliferative index as measured by PCNA staining as well as an increase in intratumoral apoptosis as measured by the TUNEL assay. This effect was strongest for groups treated with A12, either alone or in combination, which is in agreement with previous work showing only moderate apoptosis in response to cetuximab treatment alone but synergism with dual agent therapy. The increase in intratumoral apoptosis and decrease in proliferation is in contrast to our *in vitro* findings showing limited effects of either agent or the combination on the growth of Colo16 cells. The differences between the results from the animal model and *in vitro* experiments can be explained by reports showing the activation of vascular endothelial growth factor receptor (VEGFR) signaling by IGFR in cancer³⁹.

Recent studies have demonstrated that both EGFR and IGF-IR are abundantly expressed on endothelial cells^{5, 40–43}. EGF and IGF produced at high levels by tumors are able to promote the growth, survival and migration of tumor cells, as well as induce the synthesis of VEGF-A, VEGF-C and MMP2, which may enhance the development of the blood supply essential for the progressive growth of primary malignancies and their metastases^{21, 44–46}. Treatment with either A12 or cetuximab alone resulted in statistically significant inhibition of tumor associated angiogenesis while the combination treatment with A12 and cetuximab resulted in an additional inhibition of angiogenesis (Fig 6A, C). These findings concur with

other studies that have demonstrated reduction of angiogenesis resulting from inhibition of IGF-IR, EGFR, or both receptors simultaneously ^{5, 45}.

Current studies have indicated that the efficacy of tyrosine kinase inhibition can be enhanced by combining it with other tyrosine kinase inhibitors, chemotherapy, or radiotherapy^{45, 47–50}. Although a demonstrable response was achieved using A12 or cetuximab as single agents, the enhanced response obtained when these two monoclonal antibodies were used in combination provides further support for the use of not only multiple tyrosine kinase inhibitors, but the use of inhibitors of IGF-IR and EGFR in particular^{51–54}. This agrees with previous working showing a synergistic effect of the combination of IGF-IR and EGFR inhibition and suggests the presence of “cross-talk” between the receptors³³.

In summary, dual inhibition of the tyrosine kinases EGFR and IGF-IR can decrease skin cancer growth both *in vitro* and *in vivo*. Administration of the IGF-IR antibody A12 can significantly inhibit the proliferation of SCC cells *in vitro*. Treatment with A12 alone or in combination with cetuximab significantly reduces tumor volume and prolongs the survival time of nude mice implanted with CSCC by enhancing their respective cytostatic effects and inducing anti-angiogenesis. These data suggest that dual inhibition of tyrosine kinases, EGFR and IGF-IR in particular, may be therapeutically useful and provide a promising strategy for the treatment of patients with aggressive CSCC.

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Figure 1. Effect of A12 and cetuximab on the inhibition of phosphorylation of IGF-IR and EGFR *in vitro*

A. Colo16. EGFR and IGF-IR phosphorylation were inhibited by C225 and A12, respectively. Total IGF-IR levels decreased with combined treatment. B. SRB1. EGFR and IGF-IR phosphorylation were inhibited by C225 and A12, respectively. No change was seen in total protein levels.

Figure 2. Combined treatment with A12 and cetuximab inhibits the growth of CSCC cell lines *in vitro*

A. MTT assay was performed on each of the three SCC cell lines (Colo16, SRB12, SRB1) in the presence and absence of A12. No significant difference in growth was identified. B. Combined treatment with A12 and cetuximab decreased the growth of Colo16 ($p < 0.001$).

Figure 3. Combination treatment with A12 and cetuximab induces apoptosis in CSCC cell lines
There was no change in the rate of apoptosis for Colo16 cells treated with A12 alone.
Combination treatment increased the percentage of apoptotic cells.

Figure 4. A12 and Cetuximab decrease the growth of cutaneous squamous cell carcinoma xenografts and improve survival in an athymic nude mouse model

A. A12 or cetuximab treatment alone inhibited CSCC xenograft growth by 51% ($p=0.058$) and 49% ($p=0.041$), respectively. Combined treatment with A12 and cetuximab inhibited SCC xenograft growth to an even greater extent, reducing mean tumor volume by 92% ($p<0.001$) B. Treatment with either A12 or cetuximab alone prolonged survival. The group treated with the combination of A12 and cetuximab demonstrated even greater survival than either the control group ($p<0.001$) or the group treated with A12 alone ($p<0.01$).

Figure 5. Immunohistochemical analysis of Colo16 xenografts

A. Representative sections of xenografts treated with A12, cetuximab or control were stained with H & E or immunostained for expression of PCNA, TUNEL, or CD31. Quantification of the mean positive stained area are shown for PCNA (B) and CD31 (D). C. Apoptotic cells per high powered field are quantified.