Direct Evidence of Target Inhibition with Anti-VEGF, EGFR, and mTOR Therapies in a Clinical Model of Wound Healing

Jingquan Jia¹, Andrew E. Dellinger², Eric S. Weiss³, Anuradha Bulusu², Christel Rushing⁵, Haiyan Li⁴, Leigh A. Howard¹, Neal Kaplan¹, Herbert Pang²,⁵, Herbert I. Hurwitz¹, and Andrew B. Nixon¹

Abstract

Purpose: In early clinical testing, most novel targeted anticancer therapies have limited toxicities and limited efficacy, which complicates dose and schedule selection for these agents. Confirmation of target inhibition is critical for rational drug development; however, repeated tumor biopsies are often impractical and peripheral blood mononuclear cells and normal skin are often inadequate surrogates for tumor tissue. Based upon the similarities of tumor and wound stroma, we have developed a clinical dermal granulation tissue model to evaluate novel targeted therapies.

Experimental Design: A 4-mm skin punch biopsy was used to stimulate wound healing and a repeat 5-mm punch biopsy was used to harvest the resulting granulation tissue. This assay was performed at pretreatment and on-treatment evaluating four targeted therapies, bevacizumab, everolimus, erlotinib, and panitumumab, in the context of three different clinical trials. Total and phosphorylated levels VEGFR2, S6RP, and EGFR were evaluated using ELISA-based methodologies.

Results: Significant and consistent inhibition of the VEGF pathway (using VEGFR2 as the readout) was observed in granulation tissue biopsies from patients treated with bevacizumab and everolimus. In addition, significant and consistent inhibition of the mTOR pathway (using S6RP as the readout) was observed in patients treated with everolimus. Finally, significant inhibition of the EGFR pathway (using EGFR as the readout) was observed in patients treated with panitumumab, but this was not observed in patients treated with erlotinib.

Conclusions: Molecular analyses of dermal granulation tissue can be used as a convenient and quantitative pharmacodynamic biomarker platform for multiple classes of targeted therapies. Clin Cancer Res; 21(15); 3442–52. ©2015 AACR.

Introduction

For the clinical development of novel targeted therapies, it is critical to validate that targets of interest are being inhibited. Many targeted therapies may not have dose-limiting toxicities, or even common non–dose-limiting toxicities, which could otherwise guide dose and schedule selection. Furthermore, preclinical models often do not accurately reflect the human setting for a variety of reasons. A key consequence of this limitation is the difficulty of using these models to identify a specific pharmacokinetic parameter that can be used to guide dose selection in patients. Aside from dose selection, pharmacodynamic markers can also be useful to evaluate the downstream consequences of target inhibition, which may affect drug sensitivity, resistance, and toxicity.

In patients with cancer, paired pretreatment and on-treatment tumor biopsies remain the gold standard for the evaluation of target inhibition and the downstream consequences of that inhibition. However, repeat biopsies carry significant risks and costs. In addition, only a minority of patients will have tumors amendable to serial biopsies. The small size of such biopsies, variable stromal contributions, and heterogeneity within and among tumor lesions, can all complicate interpretation of tumor-based pharmacodynamic studies. For these reasons, surrogate tissues have been extensively evaluated for biomarker assessments, including plasma and serum, circulating peripheral mononuclear cells (with or without ex vivo stimulation), quiescent skin, and hair follicles. However, the relevance of these tissues is often uncertain. The assessments of skin and hair follicles are also often further limited by the amounts of tissue provided and the use of largely semiquantitative IHC methods.

To address these limitations, we have developed a dermal wound model for the assessment to targeted therapies in patients (1). This model initially uses a punch biopsy to stimulate wound healing. After one week, the wound is filled with granulation tissue, which can then be harvested with a repeated punch biopsy. This granulation tissue provides a sufficient amount of material for molecular analyses by ELISA or PCR, although each methodology needs significant optimization for these types of analyses.
Translational Relevance

Direct and quantitative evidence of target inhibition by antiangiogenic agents is needed to monitor the pharmacodynamic effects of targeted therapies in patients. In this article, we have examined the modulation of downstream molecular targets of VEGF, mTOR, and EGFR inhibitors in granulation tissue obtained at baseline and on-treatment from patients enrolled across three different clinical trials. Our results demonstrated significant and consistent inhibition of the targeted pathways after treatment in each of the clinical trials examined. Granulation tissue biopsies are less expensive and more readily accessible than tumor biopsies. These features make granulation tissue biopsies a practical and reliable tissue source for further exploration of the pharmacodynamic effects of novel targeted therapies.

addition, the surrounding dermis displays a highly reproducible pattern of vascularization that can be quantified, consistent with the role of angiogenesis in wound healing.

This model is safe, convenient, low-cost, and can be used repeatedly in the context of most clinical trials. Granulation tissue, as opposed to quiescent skin, provides a potentially clinically relevant surrogate tissue because tumor stroma and wound stroma exhibit many similarities, a topic which has been extensively reviewed (2). Indeed, tumors have been compared with "wounds that do not heal" (3). Granulation tissue is highly proliferative and angiogenic, which makes this approach particularly well suited for drugs with antiangiogenic properties. Wound healing and skin toxicities have been reported for many targeted therapies, including VEGF, mTOR, and EGFR inhibitors (4, 5).

Previously, we have shown that this model could detect the antiangiogenic effects of multiple antiangiogenic agents (6). We have further optimized the methods for imaging the dermis around the wound and developed new methods to allow characterization of key signaling proteins to be performed on the harvested granulation tissue. This report describes the feasibility of using this wound model to investigate the pharmacodynamic modulation of key antiangiogenic protein targets in response to a several distinct therapies. Although we have shown that our model can be used in a semiquantitative manner to evaluate wound angiogenesis, we now show that the dermal granulation tissue can be used for quantitative assessment of key signaling pathways under physiologic conditions and during treatment with targeted agents. Here, we evaluate this model in a series of three related phase I and II clinical trials that evaluated doublet and triplet combinations of either bevacizumab, everolimus, erlotinib, or panitumumab, agents that target the VEGF, mTOR, and EGFR, signaling pathways, respectively. Biomarker substudies in these trials were specifically designed to help assess the pharmacodynamic impact of each of these drugs on their respective targets.

Patients and Methods

Clinical trials

The parent phase I and phase II clinical trials that included the current biomarker substudies have been previously reported (7–9); characteristics of those patients who participated in the biomarker studies and details of the treatment regimens are listed in Supplementary Tables S1–S3, as well as in Fig. 1. All patients participating in these biomarker studies were treated at Duke University Medical Center (Durham, NC) and signed informed consent for the therapeutic study, which included the described biomarker analyses. All studies were approved by the Duke University Medical Center Institutional Review Board and the Duke Comprehensive Cancer Center Protocol Review Committee, and were registered on clinicaltrials.gov. The eligibility criteria for the parent treatment studies have previously been reported. The doses for each agent used in this biomarker study are listed in Supplementary Tables S2 and S3. Bevacizumab was dosed at its standard higher dose (10 mg/kg i.v. every 2 weeks) in all patients. Everolimus was dosed at its standard dose of 10 mg orally daily when combined with bevacizumab. Because of increased levels of rash and mucositis seen with anti-mTOR and anti-EGFR combinations, when these agents were combined in these studies, the doses of everolimus (5 mg daily or 5 mg three times per week), erlotinib (75 mg orally daily), and panitumumab (4.8 mg/kg i.v. every 2 weeks) were below their respective monotherapy doses, although these doses remained within the standard dose reductions used for these agents. Sequential skin biopsies were required for each of these treatment studies unless the patient had a contraindication to a skin biopsy (such as active skin infection or skin condition) or where drug toxicity, disease progression, or inter-current illness lead to a treatment interruption that would have compromised the biomarker analysis.

Skin biopsy procedures and vascular scoring. Skin biopsies were obtained using previously reported methods (1). Briefly, before treatment, a 4-mm skin punch biopsy (Fray Products Corp.) was created to stimulate granulation tissue formation followed by a 5-mm biopsy of the healing wound (granulation tissue) collected 7 days later. Briefly, the patient’s skin was locally anesthetized with 1% lidocaine with epinephrine at a ratio of 1:100,000 mixed with sodium bicarbonate (8.4%) followed by either a 4-mm stimulation or 5-mm granulation punch biopsy. Topical antibiotic ointment followed by a nonocclusive bandage was applied to each wound. Tissue biopsies were embedded in optimal cutting temperature (OCT) compound and then immediately snap frozen in liquid nitrogen and stored at −80°C. Patients were given written and verbal wound care instructions. The granulation tissue biopsies (5 mm) were harvested at baseline (before any treatment) and after at least one week of treatment with the respective targeted agent(s). The time points for each biopsy are listed in Fig. 1. On treatment time-points were expected to reflect near steady state levels of each drug while minimizing patient travel and treatment delays. Dermal neovascularization at the wound periphery was evaluated using a digital camera with a special dermatologic adapter (Heine Dermatophote). All of the images were scored using an ordinal 0–4 scoring system of wound vascularization, with each wound field divided into 12 clock hours. Each image was scored by two independent observers blinded to treatment status and image timing, and average vascular scores (AVS) were obtained for each time point (1).

Protein extraction from granulation tissue biopsies. Tissue samples were thawed at room temperature and removed from the OCT once softened. The samples were washed in ice-cold PBS and immediately transferred to Fast Prep Homogenization tubes preloaded with ice-cold lysis buffer, a modified RIPA buffer...
containing 20 mmol/L HEPES, 154 mmol/L NaCl, 2 mmol/L 
MgCl₂, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 
proteinase inhibitor cocktail (Roche, cat# 04693150001), and 
phosphatase inhibitor cocktails I and II (Sigma, cat# P2850 and 
P5726). Tissues were homogenized using Bio101 Thermo 
Savant FastPrep FP120 (Qbiogene, Inc) tissue homogenizer at 
4°C. Lysates were subsequently centrifuged at 16,000 
g at 4°C to remove unbroken cells, nuclei, and other particulates.

Protein analysis
Protein lysates were analyzed using the Meso Scale Discovery 
platform (MSD). Human KDR kit (Cat# K151BOC) and Phospho 
(Tyr 1054) VEGFR2 Whole Cell Lysate kit (Cat# K151DJ), 
Phospho(Ser240/Ser244)/Total S6RP Whole Cell Lysate kit (Cat# 
K15139), and Phospho (Tyr1173)/Total EGFR Whole Cell Lysate 
kit (Cat# K15104D), were used to evaluate VEGFR2, mTOR, and 
EGFR pathways. All assays were performed in duplicate using 50 
and 100 μg of total protein according to manufacturer’s recom-
mendations and read on MSD sector imager 2400 instrument 
(MSD, Cat# R92TC-2).

Statistical analysis
Frequency tables were generated for the demographic vari-
ables and median and range were provided for the relevant 
variables. To evaluate on-treatment changes, L-ratio was calcu-
lated using the formula Log₂ (posttreatment level/baseline 
level) for each marker. To determine the significance of L-ratio 
changes, Wilcoxon signed rank tests were used to analyze 
changes in total VEGFR2, phospho-VEGFR2, total EGFR, phos-
pho(Tyr1173) EGFR, total S6RP, phospho(Ser240/Ser244) 
S6RP expression, as well as AVS. Waterfall plots for on-treat-
ment changes were illustrated. Boxplots for percentage change

Figure 1.
Timeline of treatment and granulation tissue biopsy acquisition in each study.
from baselines were illustrated. Results with significant $P$ values were further corrected for multiple testing with Benjamini–Hochberg FDR. The correlation between (i) the change in analyte levels of the various treatment regimens and (ii) AVS and inhibition of VEGFR2 was evaluated using Spearman’s rank correlation.

Results

Patient characteristics and study cohort description

The number of the patients with molecular biomarker analysis and their demographics across each study are summarized in Supplementary Table S1. No significant wound-healing complications from wound biopsies occurred on any of these trials.

In the phase I BEE study, a total of 38 patients with a variety of solid tumors were evaluable for biomarker analysis. These patients were treated on four biomarker cohorts: (i) everolimus monotherapy for 2 weeks before the addition of bevacizumab and erlotinib ("Ev-BEE" cohort, $n = 9$); (ii) erlotinib monotherapy for 2 weeks before the addition of bevacizumab and everolimus ("Erl-BEE" cohort, $n = 9$); (iii) bevacizumab plus everolimus doublet therapy ("BE" cohort, $n = 15$); and (iv) bevacizumab plus everolimus plus erlotinib triple therapy ("BEE" cohort, $n = 5$). Full-thickness dermal granulation tissue biopsies were taken at baseline, end of the monotherapy lead-in period (day 14), and 35 days post-doublet or triplet therapy. All patients who received bevacizumab, everolimus, and erlotinib treatments concurrently were analyzed as one group, referred to as "Bev+Ev+Erl," whether they received this triplet therapy initially ($n = 5$), or whether the triplet was received after everolimus monotherapy ($n = 8$) and erlotinib monotherapy ($n = 7$) lead-ins.

In the phase I BEP study, a total of 18 patients with a variety of solid tumors were evaluable for biomarker analysis. These patients were treated in four biomarker cohorts: everolimus monotherapy for 2 weeks before the addition of bevacizumab and panitumumab ("Ev-BEP" cohort, $n = 1$), bevacizumab plus everolimus doublet therapy for 2 weeks before the addition of panitumumab ("BE-BEP" cohort, $n = 5$), everolimus plus panitumumab doublet therapy for 2 weeks before the addition of bevacizumab ("EP-BEP" cohort, $n = 4$), bevacizumab plus everolimus plus panitumumab triple therapy ("BEP" cohort, $n = 8$). For those cohorts who received a monotherapy or doublet therapy lead-in, dermal granulation tissue biopsies were taken at baseline, the end of lead-in therapy (day 14), and 42 days after BEP triplet therapy. For the "BEP" cohort, dermal granulation tissue biopsies were taken at baseline and 28 days posttreatment. Because of the small number of patients available for biomarker analysis in each cohort of the BEP study, the lead-in time point was not analyzed. All patients who received bevacizumab, everolimus, and panitumumab treatments concurrently were analyzed as one group. This includes 6 patients in the "BEP" cohort, 3 patients in the "BE-BEP" cohort, 2 patients in the "EP-BEP" cohort, and 1 patient in the "Ev-BEP" cohort.

In the phase II Bev-Ev study, 40 patients with refractory metastatic colorectal cancer were evaluable for biomarker analyses. All patients received bevacizumab plus everolimus doublet therapy; there was no monotherapy component or cohort in this study. Dermal granulation tissue biopsies were taken at baseline and 28 days posttreatment. The time points for drug administration and biopsy collection for these three clinical trials are summarized in Fig. 1.

Inhibition of total and phospho VEGFR2 protein

Total VEGFR2 levels were significantly reduced by bevacizumab treatment given in combination with everolimus (Fig. 2C and 2F), with everolimus plus erlotinib (Fig. 2D), and with everolimus plus panitumumab (Fig. 2E; $P < 0.05$). Phosphorylation of VEGFR2 was also significantly inhibited by bevacizumab treatment given in combination with everolimus (Fig. 3F), and in combination with everolimus plus erlotinib (Fig. 3D). However, phospho VEGFR2 was not significantly inhibited by Bev+Ev+Pmb treatment (Fig. 3E). Because of the concern of the small sample size of each treatment group and therefore limited statistical power, a combined analysis was done using data pooled from all three trials where every patient who had received bevacizumab during the course of the study was analyzed together as one group, referred to as “All Bev Pts.” For those patients who have received bevacizumab from the lead-in phase, only the final on-treatment biopsy was included for the statistical analysis. Both total and phospho VEGFR2 were significantly inhibited in the “All Bev Pts” group (Figs. 2G and 3G; $P < 0.001$), and the average percent inhibition was approximately 50% for total VEGFR2 and 25% for phospho VEGFR2 (Figs. 2H and 3H). Even though the VEGFR2 assays were normalized to total protein, the specific cellular composition of the biopsy samples were not measured due to the limited size of the biopsies; thus these experiments cannot determine whether the reductions in total VEGFR2 might be due a reduction in the number of endothelial cells, a reduction in VEGFR2 production per endothelial cell, or both mechanisms. Monotherapy treatment with everolimus, which is known to have potent antiangiogenic activity, also led to a significant loss in total VEGFR2 expression (Fig. 2A; $P = 0.002$). However, phospho VEGFR2 levels were not significantly downregulated by everolimus monotherapy (Fig. 3A). Erlotinib monotherapy did not appear to affect VEGFR2 expression or phosphorylation (Figs. 2B and 3B).

Taken together, these data demonstrate direct target inhibition of VEGFR2 by the combination of bevacizumab and everolimus treatment. This combination decreased receptor expression and decreased the phosphorylation state of the receptor. Although everolimus monotherapy reduced total VEGFR2 expression, the combination of bevacizumab plus everolimus did not appear to increase the level of inhibition over that seen with everolimus treatment alone (Fig. 2H).

Inhibition of total and phospho S6RP protein expression

p70S6 Kinase (S6K1) and 4EBP1 are two well-characterized downstream effectors of mTOR activity. Activated S6K1 phosphorylates S6 ribosomal protein (S6RP) to promote mRNA translation (10). The level of phosphorylated (Ser240/244) S6RP (pS6RP) has been shown to reflect the activation of S6K1 (11). Preclinical studies suggested that S6RP is a reliable measure of mTOR blockade (12, 13). For these reasons, total and phospho S6RP (Ser240/244) S6RP protein levels were used as markers of mTOR inhibition.

Everolimus monotherapy did not significantly change total S6RP protein expression (Fig. 4A). Erlotinib alone did not have a significant effect on either total (Fig. 4B) or phospho levels of S6RP (Fig. 4B). However, total S6RP protein expression was significantly decreased by everolimus treatment in combination with bevacizumab (Fig. 4E), with bevacizumab plus erlotinib (Fig. 4D) or with bevacizumab plus panitumumab (Fig. 4E;
As a direct target of mTOR pathway activity, phospho S6RP protein levels were significantly reduced by everolimus treatment given alone (Fig. 5A), in combination with bevacizumab (Fig. 5C and 5F), in combination with bevacizumab plus erlotinib (Fig. 5D), or in combination with bevacizumab plus panitumumab (Fig. 5E; $P < 0.05$). All patients who received everolimus treatment on one of the three clinical trials were also evaluated as one group, referred to as "All Ev Pts." Both total and phospho S6RP protein expressions were significantly reduced (Figs. 4G and 5G), and the average percent inhibition observed was about 50% for total S6RP and 85% for phospho S6RP (Figs. 4H and 5H). Taken together, our data demonstrate direct target inhibition of the mTOR pathway by everolimus treatment, either alone or in combination with bevacizumab and EGFR inhibitors.

**Inhibition of total and phospho EGFR protein expression**

Total EGFR (tEGFR) protein levels and phosphorylated (Tyr 1173) EGFR (pEGFR) were evaluated to monitor the activity of erlotinib and panitumumab in the granulation tissue model. Interestingly, erlotinib monotherapy did not appear to significantly reduce either total EGFR or phospho EGFR levels, although our study was not powered to detect modest levels of inhibition (Fig. 6B and 6F). Similarly, no significant decrease in total or phospho EGFR protein levels was observed with erlotinib treatment in combination with bevacizumab and everolimus (Fig. 6C.


Figure 2.

Changes in total VEGFR2 levels from baseline. A–G, each waterfall plot represents a specific treatment regimen in each trial. The $y$-axis represents the log fold change in total VEGFR2 protein level from baseline. Each line along $x$-axis represents an individual patient. H, percent change in total VEGFR2 protein levels from baseline in response to the different treatment regimens. Ev, everolimus monotherapy; Erl, erlotinib monotherapy; Be, bevacizumab; BEE, bevacizumab + everolimus double therapy; BEP, bevacizumab + erlotinib + panitumumab triple therapy; BE_BEP, BE and BEP regimen combined; Pooled, all treatment regimen combined.
However, panitumumab treatment, in combination with bevacizumab and everolimus, significantly inhibited both total and phospho EGFR levels (Fig. 6D and 6H; $P < 0.001$). This result is consistent with the fact that panitumumab competitively inhibits EGFR ligand binding, promotes receptor internalization, and leads to tyrosine kinase phosphorylation (14).

In addition, we examined the correlation across the change in analyte levels after the various treatment regimens. We compared all phospho proteins among themselves, and all total proteins among themselves. Spearman’s rank correlations were calculated and $P$ values were Bonferroni multiple testing corrected. All markers were positively correlated with one another, as all correlations had a positive rho value (correlation coefficient). After Bonferroni correction, we were able to observe a significant correlation between tS6RP and tVEGFR2 ($P < 0.0001$) in the Bev–Ev study. Furthermore, significant correlations were also noted between pEGFR and pS6RP ($P = 0.0018$) and tEGFR and tVEGFR2 ($P = 0.0072$) in the BEE study.

Average vascular score in dermal wound tissue

As a physiologic response to a wound, the dermis around the punch biopsy undergoes an ordered series of vascular responses, which can be reproducibly measured (1). Wounds are imaged at a specific time point and subsequently divided into 12 distinct...
regions that are individually scored. The resulting AVS across all regions is then calculated (6). AVS was assessed one week after the wound stimulus, just before the granulation tissue being harvested for molecular analyses. AVS data from matched sets of pretreatment and on-treatment images were available for 22 patients on the BEE study, for 24 patients on the BEP study, and 36 patients on the BE study.

Patients treated on the BE study exhibited AVS scores that were significantly decreased by bevacizumab treatment in combination with everolimus ($P = 0.009$; Supplementary Fig. S1A). Of the 36 evaluable patients, 25 patients had decreased AVS scores after bevacizumab and everolimus. Patients treated on the BE study also exhibited AVS scores that were significantly decreased (18 out of 24 evaluable patients) by bevacizumab treatment in combination with everolimus and panitumumab ($P = 0.023$; Supplementary Fig. S1B). Although not statistically significant, a trend for a decrease in AVS score after BEE triplet therapy was noted ($P = 0.098$; Supplementary Fig. S1C).

**Discussion**

For essentially all novel targeted therapies, reliable pharmacodynamic assays are critical for optimal dose selection and to understand the downstream consequences of target inhibition.
These assays need to be pragmatic, quantitative, and amenable to assessments at both pretreatment and on-treatment. These assays should also be fit for purpose and reflect the known biology of the target.

The current set of biomarker studies further demonstrates the feasibility of using our dermal wound model. We confirmed that our average vascular scoring analysis (AVS) provides semiquantifiable measures for overall vascularization of the wound and that antiangiogenic agents inhibit this process. In this report, we demonstrate that molecular analysis of granulation tissue can provide a quantitative assessment of target inhibition for multiple targeted therapies, provided the targets are readily expressed in granulation tissue. Inhibition of VEGFR2 was demonstrated for bevacizumab, inhibition of mTOR for everolimus, and inhibition of EGFR for panitumumab. In this analysis, the model appears to be sensitive enough to detect these effects in a limited number of patients and robust enough to demonstrate these effects across three different trials with different targeted therapies. This model was also quantitative enough to detect potentially meaningful differences in the levels of target inhibition.

These current biomarker studies are among the first to demonstrate direct target inhibition for a VEGF inhibitor.

Figure 5. Changes in phospho S6RP levels from baseline. A–G, each waterfall plot represents a specific treatment regimen in each trial. Y-axis represents the log fold change in phospho S6RP protein level from baseline. Each line along x-axis represents an individual patient. H, percent change in phospho S6RP protein level from baseline in response to the different treatment regimens. Ev, everolimus monotherapy; Erl, erlotinib monotherapy; BE, bevacizumab + everolimus double therapy; BEE, bevacizumab + everolimus + erlotinib triple therapy; BEP, bevacizumab + everolimus + panitumumab triple therapy; BEE_BEP, all treatment regimen combined.

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inhibition of VEGFR2 phosphorylation by bevacizumab in granulation tissue was consistently seen across our trials and across cohorts within trials. Furthermore, a reduction in total VEGFR2 levels in granulation tissue was highly correlated with changes in vascularization in the dermis at the wound periphery. The ability to correlate molecular and physiologic changes in angiogenesis may be particularly important for the evaluation of novel antiangiogenesis agents and combination antiangiogenic regimens. These data also suggest that this model may be useful to better understand the pathophysiology of these agents on wound healing in humans. Importantly, there were no significant wound-healing complications from the biopsies done on these studies and all wounds did heal, although many had significant delays. Because cancers are known to co-opt many wound-healing programs (15, 16), these findings support the potential relevance of our dermal wound model for studying the mechanisms of action, toxicity, and resistance for some targeted therapies.

Previous biomarker studies with bevacizumab have noted changes in vascular density (17) and vascular maturity (18), as well as changes in phosphorylated VEGFR2 (19). These important studies, however, relied upon IHC methods and needed to enroll select patient populations. Our results are consistent with those reports and the methods used here are more readily adapted to the
broad populations that comprise most dose finding phase I studies. We were also able to demonstrate target pathway inhibition for the mTOR inhibitor everolimus. The mTOR pathway is complex, with multiple signaling nodes and feedback loops, including HIF1α and IGF1R (20). Although we were able to reliably analyze S6RP, a known downstream effector of mTOR, we did not analyze additional potential effectors due to the technical limitations related to available reagents and the small size of granulation tissue samples. This effect on S6RP was consistent across different studies and cohorts, even though everolimus doses were significantly reduced when combined with bevacizumab, panitumumab, or erlotinib. mTOR inhibitors have antiangiogenic properties and have been associated with wound healing complications (21); the loss of total VEGFR2 on everolimus detected in our model was consistent with a reduction in endothelial cell number on treatment.

EGFR inhibition was observed in our analysis of the anti-EGFR antibody, panitumumab, but not the anti-EGFR tyrosine kinase inhibitor, erlotinib. The doses of erlotinib and panitumumab used in most patients in our studies were 50% and 75% of their typical monotherapy doses, respectively. The lack of detectable EGFR inhibition with erlotinib may reflect the lower doses used in these studies. These differences may also reflect mechanistic differences between monoclonal antibody and TKI inhibition of EGFR. The severity of skin rash has been noted to differ among erlotinib, cetuximab, and panitumumab (22). Our data suggest, that these differences could be related to different levels of EGFR inhibition. Additional studies, particularly head to head comparisons of monotherapy treatments, would be needed to confirm any differences between these agents or between different doses with respect to target inhibition and downstream effects. Our model provides a convenient and quantitative platform for such comparisons. It should be pointed out that this report has several limitations. Our biomarker substudy analyses were implemented into the treatment regimens and were explored based on the feasibility of integrating this approach into traditional phase I and II testing. In this context, testing of all treatment monotherapies and combinations was not possible. For this reason, we focused on the cohorts of greatest interest. Similarly, sample sizes were driven by the clinical needs of the study and were thus based upon pragmatic clinical considerations, not formal power calculations around biomarkers. Nevertheless, our results were remarkably consistent across three separate studies. Finally, we did not perform simultaneous tumor biopsies in these studies to directly compare findings in tumors and wounds. Although tumors have been compared with wounds that do not heal, all tumors are not the same, nor are all wounds. This fact emphasizes the need for understanding the similarities and differences in tumor and wound tissues and the need to apply this assay, like all surrogate biomarker assays, in a fit for purpose framework. Nevertheless, our findings are consistent with numerous molecular comparisons that have shown numerous similarities between tumors and wounds (23–26).

In conclusion, our dermal wound model provides direct evidence for target inhibition of VEGFR2 by bevacizumab, mTOR by everolimus, and EGFR by panitumumab. These results were consistent across several phase I and II studies. This dermal wound model may also be useful to evaluate other novel targeted therapies and combinations.

Disclosure of Potential Conflicts of Interest

H. Hurwitz is a consultant/advisory board member for Amgen, Genentech/Roche, and Lilly/Imclone, and reports receiving commercial research grants from Amgen, Bristol-Myers Squibb, Genentech/Roche, Lilly/Imclone, and Novartis. A. Nixon is a consultant/advisory board member for Genentech/SmithKline and Novartis, and reports receiving commercial research grants from Amgen, Pfizer, and Tracor Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: H. Hurwitz, A.B. Nixon
Development of methodology: J. Jia, H. Hurwitz, A.B. Nixon
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Jia, E. Weiss, L. Howard, N. Kaplan, H. Hurwitz
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Jia, A. Dellinger, A. Bulusu, E. Weiss, E. Pang, H. Hurwitz, A.B. Nixon
Writing, review, and/or revision of the manuscript: J. Jia, A. Dellinger, E. Weiss, C. Rushing, H. Pang, H. Hurwitz, A.B. Nixon
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Dellinger, E. Weiss, H. Li, N. Kaplan, H. Hurwitz
Study supervision: H. Hurwitz, A.B. Nixon

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References


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