Published in final edited form as:

Head Neck. 2013 January ; 35(1): 86–93. doi:10.1002/hed.22936.

The Effects of Epidermal Growth Factor Receptor and Insulin-like Growth Factor 1 Receptor Inhibition On Proliferation and Intracellular Signaling In cSCCHN: Potential for Dual Inhibition As a Therapeutic Modality

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Abstract

Background—Combined inhibition of epidermal growth factor receptor (EGFR) and insulin-like growth factor receptor 1 (IGF1R) has been proposed as a therapy for cutaneous squamous cell carcinoma of the head and neck (cSCCHN).

Methods—Receptor expression and downstream signaling was assessed in cSCC cell lines and patient samples. EGFR and IGF1R signaling was inhibited in cSCC cell lines using erlotinib and/or picropodophyllin

Results—EGFR and IGF1R were overexpressed in cSCCHN specimens relative to normal skin. Dual inhibition of both receptors prevented cell growth and decreased activation of Akt and p42/44 MAPK more effectively than either inhibitor alone.

Conclusion—Dual inhibition of EGFR and IGF1R is effective at blocking cell growth, and is correlated with inhibition of Akt and p42/44 MAPK, suggesting that this may be a promising treatment for cSCCHN.

Keywords

Epidermal growth factor receptor; Insulin-like growth factor receptor; cutaneous squamous cell carcinoma

Introduction

Over 200,000 cases of cutaneous squamous cell carcinoma (cSCC) are diagnosed each year in the United States, many of which are found on the head and neck (cSCCHN).¹ While most are early stage tumors and are easily treated with surgical excision, approximately 5% of patients will develop metastases.² Unfortunately, ten year survival rates are <20% with lymph node metastases and <10% with distant metastases.¹ This is likely the result of patient factors, such as frequent medical comorbidities, and treatment factors including a

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relatively poor understanding of the molecular underpinnings of metastatic cSCC and the overall poor responsiveness of this disease to traditional therapies.^{3,4} Thus, there is increasing interest in applying targeted molecular agents for treatment of cSCC.

Epidermal growth factor receptor (EGFR) has emerged as one potential therapeutic target for cSCCHN.⁵ EGFR is upregulated in 80–100% of cSCC^{6,7} and is linked to carcinogenesis in skin and in other organs.⁸ Given the effectiveness of selective EGFR inhibitors in other EGFR-expressing tumors^{9–11}, EGFR inhibitors such as erlotinib, a small molecule tyrosine kinase inhibitor, and cetuximab, a monoclonal blocking antibody, have been used in patients with metastatic cSCCHN on an off-label basis and in early phase clinical trials. However, to date there are no Phase III studies directly examining their effectiveness in this patient population.

Although EGFR inhibitors have shown promise in a variety of tumor types, their utility is often limited by the rapid development of resistance.¹² In other tumor types, resistance to EGFR inhibitors has been shown to occur through increased signaling via insulin-like growth factor 1 receptor (IGF1R).^{13–16} Subsequent inhibition of IGF1R can overcome resistance to EGFR inhibitors. Recently, dual inhibition of EGFR and IGF1R using blocking antibodies was shown to prevent the growth of human cSCC cells in culture and in mouse xenografts models¹⁷. However, no *in vivo* data exists from patient specimens to indicate that IGF1R is involved in cSCC resistance to EGFR inhibitors or that inhibition of IGF1R in combination with EGFR may benefit patients with advanced cSCC. Furthermore, the effectiveness of small molecule EGFR inhibitors, rather than monoclonal blocking antibodies, has not yet been established in cSCCHN. Small molecule IGF1R inhibitors are currently being investigated. One such inhibitor, picropodophyllin (PPP), is well tolerated *in vivo*; in some animal studies it has been tolerated for several months of daily treatment without any adverse effects noted¹⁸. It is highly specific for IGF1R and does not significantly inhibit the insulin receptor, a potential dose-limiting effect of less specific IGF1R inhibitors¹⁹. While limited human data exist to confirm the importance of avoiding inhibition of the insulin receptor, PPP has shown utility in treating several tumor types in experimental systems including uveal melanoma^{20,21}, multiple myeloma¹⁸, osteosarcoma²², and glioblastoma²³. In this study we aimed to explore the role of EGFR and IGF1R signaling pathways in human cSCCHN and to assess the effect of dual inhibition of IGF1R and EGFR with small molecule tyrosine kinase inhibitors on cSCC.

Materials and Methods

Cell Lines and Reagents

SCC12 and SCC13 cell lines, derived from head and neck primary cSCC, were obtained from Dr. James Rheinwald.²⁴ Creation of the MET1 and MET4 cell lines, derived from a forearm cSCC and its associated lymph node metastasis, respectively, has been previously described.²⁵ All cells were grown in HDMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS in a humidified 5% CO₂ incubator at 37°C. Erlotinib was purchased from Selleck Chemicals (Houston, TX, USA). Picropodophyllin was purchased from Enzo Life Sciences (Farmingdale, NY, USA).

Cell Proliferation Assays

MET1, MET4, SCC12, or SCC13 cells were plated at a density of 5,000 cells per well in 96-well plates. Varying concentrations of erlotinib or PPP spanning physiologic doses previously shown to inhibit other tumor types in cell culture were added and cells were cultured for 48 hours.^{12,26,27} (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays (Promega, Madison WI, USA) were performed in triplicate, according to

manufacturer's protocol. The absorbance at 570nm in each well was measured using a Spectramax Plus plate reader (Molecular Devices, Sunnyvale, CA, USA).

Receptor Phosphorylation Status by ELISA Assay

MET1, MET4, SCC12, and SCC13 cells were individually plated at a density of 50,000 cells per well in 6-well plates. Vehicle control, 500nM erlotinib, 150nM PPP, or both inhibitors were added to the cells. The cells were grown for 48 hours, then harvested into DuoSet IC lysis buffer (R&D systems, Minneapolis, MN, USA). ELISA assays were then performed according to the manufacturer's protocol. The absorbance of each well at 450nm was determined using a Spectramax Plus plate reader (Molecular Devices). All assays were performed in triplicate.

Signaling Assays

MET1, MET4, SCC12, and SCC13 cells were grown as described for ELISA, but were harvested into Laemmli protein sample buffer. Samples were run on SDS-PAGE gels, transferred to nitrocellulose membranes, then incubated with primary antibodies. The following antibodies were used at the listed concentrations, all from Cell Signaling (Danvers, MA, USA): EGF receptor (1:1000), phospho-EGF receptor (1:1000), IGF-1 receptor β subunit (1:1000), phospho-IGF-1 receptor β subunit (1:2000), Akt (1:2000), Ser473 phospho-Akt (1:1000), p44.42 MAPK (1:1000), Thr202/Tyr204 phospho-p44/42 MAPK (1:1000), mTOR (1:2000), Ser2448 phospho-mTOR (1:1000). The membranes were washed, and incubated with either Alexa Fluor 680 anti-mouse (Molecular Probes, Carlsbad, CA, USA) or Fluor 800 anti-rabbit (Li-Cor, Lincoln, NE, USA) then imaged using a Li-Cor Odyssey Scanner (Li-Cor). Quantification was performed using Li-Cor analysis software (Li-Cor) and expression levels were normalized to α -tubulin.

Patient Samples

Patients treated with cSCC at Oregon Health and Science University in the departments of Dermatology and Otolaryngology/Head and Neck Surgery over the last 10 years have been enrolled in a research tumor bank. This tissue bank also houses normal skin specimens. This tumor bank and associated studies were approved by the Oregon Health and Science University institutional review board. Nine cSCCHN and normal-appearing skin specimens from the same patients were selected for use in this study.

Immunofluorescence

Formalin-fixed, paraffin-embedded, 5 μ m-thick tissues sections were evaluated with antibodies using standard conditions²⁸ and antigen retrieval (10 mmol/L citrate buffer, pH 6, or 10mmol/L Tris/1 mmol/L ethylenediaminetetraaceticacid [EDTA], pH 9, at 100°C for 50 minutes). Tissue sections were incubated with the following primary antibodies overnight at 4°C at 1:300 dilution: EGF receptor (Cell Signaling), IGF1 receptor (Santa Cruz Biotechnology, Santa Cruz, CA), and pan-cytokeratin (Fitzgerald, Acton, MA). Visualization was facilitated by fluorescent detection of Cy5-conjugated (1:1000; Jackson ImmunoResearch, West Grove, PA), Alex Fluor 546-conjugated (1:1000; Molecular Probes), or Alexa Fluor 488-conjugated (1:1000; Molecular Probes) secondary antibodies and the nuclear stain Hoescht 33258 (100ng/ml; Sigma-Aldrich, St. Louis, MO) using a Leica confocal microscope (Leica Microsystems, Wetzlar, Germany) and identical settings across all slides.

Quantification of Fluorescence

Immunofluorescent images were captured with separate lasers using fluoview FV1000 software (Olympus, Center Valley, PA). CellProfiler²⁹ software was used to determine

expression of EGFR and IGF1R in cSCC cells. Briefly, the software was configured using a reference image to identify cytokeratin-positive cells in the image as cSCC cells. Thresholds were then set for positive EGFR or IGF1R expression such that the CellProfiler correctly identified cells within the reference image that a trained observer would also score as positively expressing cells. All images were subsequently processed using the defined thresholds and the percentage of cytokeratin-positive cells expressing either EGFR or IGF1R was reported.

Statistics

ANOVA and Student's t test were performed using GraphPad Prism (GraphPad Software, San Diego, CA) to determine statistical significance with a $p < 0.05$ indicating significance. Erlotinib and PPP IC50 values for all cell lines were determined by curve fitting MTT assay absorbance values across the full range of inhibitor concentrations tested using Kaledagraph (Synergy Software, Redding, PA), then solving the resulting curve equation for a 50% response.

Results

While there is substantial evidence that EGFR is overexpressed in cSCC, there is little data examining IGF1R expression in human cSCCHN tumors. Thus, pathologic specimens of normal-appearing skin and primary cSCCHN were evaluated for EGFR and IGF1R expression by immunofluorescent analysis (Figure 1A). Consistent with previous reports³⁰, morphologic analysis showed that EGFR was primarily expressed in the basal cell layer of the epidermis in normal skin (Figure 1B). In contrast, EGFR was expressed more widely expressed in cSCCHN, with approximately threefold more expression than normal-appearing skin (Figure 1C). IGF1R was distributed throughout the epidermis at low levels in normal skin, and was upregulated approximately 50% in cSCCHN samples, including in many EGFR overexpressing cells. The overexpression of both receptors in cSCCHN suggests that dual inhibition of these receptors may be a useful chemotherapeutic strategy for cSCCHN.

Both erlotinib and PPP are designed to inhibit receptor autophosphorylation, the first step in receptor signaling. To assess the effect of these inhibitors on receptor phosphorylation, MET1 cells were cultured in the presence of erlotinib, PPP, or both inhibitors. Cell lysates from these cultures were then immunoblotted for phospho-EGFR, phospho-IGF1R, and total receptor expression (Figure 2A). Both EGFR and IGF1R were strongly expressed in the cell lysates, and were unchanged by the presence of either inhibitor. However, both phospho-EGFR and phospho-IGF1R were barely detectable by immunoblotting, making it impossible to quantitate. Similar results were seen in all cSCC cell lines (MET4, SCC12, SCC13; data not shown), consistent with previous studies of EGFR and IGF1R signaling¹⁷. Thus, ELISA assays for phospho-EGFR and phospho-IGF1R were used as a more sensitive measure of receptor phosphorylation. These demonstrated significant inhibition of EGFR phosphorylation in response to erlotinib across all cell lines (Figure 2B), and similar decreases in IGF1R phosphorylation in response to PPP (Figure 2C). Furthermore, these small molecule inhibitors were specific, with no inhibition of EGFR phosphorylation by PPP, or inhibition of IGF1R phosphorylation by erlotinib.

In order to determine the effect of erlotinib and PPP on cSCC cell growth *in vitro*, MTT assays were performed on MET1 cells grown for 48 hours in varying concentrations of erlotinib or PPP that span dose ranges previously shown to inhibit other tumor types.^{12,26,27} The number of viable cells in each culture was then compared across concentrations. Both erlotinib (Figure 3A) and PPP (Figure 3B) demonstrated dose-dependent decreases in cell proliferation with increasing concentrations, and this was also observed in MET4, SCC12,

and SCC13 cell lines (data not shown). IC50 values were determined for all cell lines; these results are shown in Table 1. Next, all cell lines were cultured for 48 hours with varying concentrations of both erlotinib and PPP in combination (Figures 3C–F). In all cases both erlotinib and PPP showed dose-dependent decreases in cell proliferation. At the highest concentrations the effect of PPP appeared to be stronger than that of erlotinib, as increasing erlotinib concentrations at high levels of PPP did not alter cell proliferation. However, at some intermediate concentrations, the combination of both inhibitors appeared to cause a greater reduction in cell proliferation than either inhibitor alone, suggesting an additive action between these two drugs (Table 2).

IGF1R signaling is hypothesized to contribute to EGFR inhibitor resistance via activation of similar downstream signaling cascades; however, multiple downstream signaling molecules have been implicated in this process. In order to determine which pathways may be relevant in cSCC, evaluation of the expression of phosphorylated and total Akt, p42/44 MAPK, and mTOR by immunoblotting was performed in cSCC cells treated either with erlotinib, PPP, or dual treatment (Figure 4A). Inhibition of Akt and p42/44 MAPK activation by both erlotinib and PPP was observed (Figures 4B and C), while no detectable change in mTOR activation occurred. Furthermore, the combination of both EGFR and IGF1R inhibitors resulted in a larger decrease in Akt and p42/44 phosphorylation than either inhibitor alone. Thus, inhibition of signaling via both the Akt and p42/44 MAPK pathways may be critical to EGFR and IGF1R inhibition of tumor proliferation.

Discussion

While early stage cSCCHN is treated well with surgical excision, patients with disease metastatic to cervical lymph nodes fare poorly³¹. There is no FDA approved chemotherapy for cSCCHN with metastases. While EGFR inhibitors have emerged as potentially promising therapeutic agents for this disease, their utility can be limited by the development of resistance. In non-small cell lung cancer, one mechanism that has been described to cause resistance to EGFR inhibitors is compensatory signaling via the IGF1R pathway^{15,16}. Dual inhibition of EGFR and IGF1R with blocking antibodies improves inhibition of tumor growth over either agent alone in cSCC.¹⁷ However, the importance of IGF1R signaling in human cSCCHN remains unclear. Thus, In this study we aimed to explore the role of EGFR and IGF1R signaling pathways in human cSCCHN and to assess the effect of dual inhibition of IGF1R and EGFR with small molecule tyrosine kinase inhibitors on cSCC.

Similar to other studies, we found EGFR to be overexpressed in cSCCHN.^{7,32} We also found IGF1R to be overexpressed in human cSCCHN samples. This is a novel finding as IGF1R overexpression was only previously demonstrated in cultured cells.¹⁷ The roughly 50% upregulation in IGF1R expression in cSCCHN relative to normal skin supports the idea that signaling via this receptor may have a role in the pathogenesis of cSCCHN and may represent a valid clinical target for anti-cSCC therapy.

While EGFR and IGF1R are readily identifiable in human tissue, our difficulty in observing EGFR and IGF1R phosphorylation in cell culture lysates using immunoblotting is not unexpected. Other studies have shown similar results in other cSCC cell lines¹⁷, suggesting that only a small fraction of the EGFR or IGF1R is active at any one time. Previous studies have demonstrated the effectiveness of EGFR and IGF1R inhibitors on receptor phosphorylation by treating cells with large doses of EGF or IGF, then applying inhibitors to block the massive increase in receptor phosphorylation that results from supraphysiologic levels of growth factors. However, we felt that artificially boosting the levels of these growth factors would not accurately reflect the *in vivo* tumor microenvironment. Using

ELISA as a more sensitive assay, we were able to show that EGFR and IGF1R inhibitors function as predicted in cSCC cells to block receptor signaling selectively.

Both erlotinib and PPP decreased cell growth in all cSCC cell lines studied. However, there was variability in susceptibility to these inhibitors across cell lines as evidenced by the range of IC50 values observed across cell lines. Our data also showed cooperation in preventing tumor growth between EGFR and IGF1R inhibitors. These findings are in agreement with other studies,^{13,14,16,17,33–35} however, additive effects of erlotinib and PPP were only observed at some intermediate concentrations, while at other concentrations there was no apparent additive effect to the use of both inhibitors. Furthermore, the addition of erlotinib to high concentrations of PPP did not alter cell growth. The reasons for this are unknown but may be due, in part, to the well-known properties of biologic agents as cytostatic rather than cytotoxic agents.^{33–36} Alternatively, this may result from the milieu of growth factors present in our cell culture media relative to that present *in vivo*. As the particular mix of growth factors present in the tumor microenvironment is currently unknown, the relative importance of each signaling pathway in cell culture may not accurately reflect the *in vivo* situation.

We found that dual inhibition of EGFR and IGF1R resulted in inhibition of downstream signaling. Specifically, we identified Akt and MAPK inhibition after treatment with EGFR and IGF1R inhibitors. This is not surprising given similar results reported after treatment with EGFR inhibitors alone. For example, in breast cancer³⁷, non-small cell lung cancer³⁸, and head and neck SCC³⁹, gefitinib was shown to decrease activation of both Akt and p42/44 MAPK. Further, cells resistant to gefitinib have increased levels of phosphorylated Akt and p42/44 MAPK. Increased Akt phosphorylation has also been linked to erlotinib resistance²⁷. In cSCC, gefitinib has been shown to inhibit p42/44 MAPK signaling⁴⁰, and EGFR inhibition decreases Akt signaling⁴¹. Inhibition of IGF1R can also prevent Akt and p42/44 MAPK phosphorylation⁴². Targeting of these signaling intermediates may also serve as a potential therapy for cSCC; indeed, a recent study showed that combined inhibition of EGFR and PI3K/Akt signaling improved growth inhibition of cSCC cells.⁴³; therefore, it is possible that the Akt and/or the p42/44 MAPK pathway serve as a common final pathway by which IGF1R activation leads to EGFR inhibitor resistance.

In summary, we have shown that EGFR and IGF1R are overexpressed in cSCC, and that dual inhibition of these receptors using tyrosine kinase inhibitors improves tumor growth inhibition. Our data suggest that either Akt or p42/44 MAPK may be the common intermediate linking these signaling pathways, although further work will be needed to decisively link Akt and/or p42/44 MAPK to EGFR and IGF1R signaling in cSCC. Together with previously published data, this provides strong support for dual EGFR and IGF1R inhibition as a new therapeutic modality for advanced cSCC and lays the foundation for future clinical trials to investigate this possibility.

Acknowledgments

This research was supported by an Oregon Health and Science University Medical Research Foundation Early Clinical Investigator grant to D.R.C.

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Figure 1. EGFR and IGF1R protein expression in cSCC

(A) Pathologic specimens of normal skin and primary cSCC were sectioned and immunofluorescence labeling performed with antibodies specific for EGFR (red) and IGF1R (yellow). Pan-cyokeratin (green) is also shown to demonstrate the distribution of cSCC cells. Nuclei were detected using DAPI staining, shown in the cyokeratin and merged images; Bar=50 μ m. EGFR and IGF1R were overexpressed in cSCC samples. (B) Schematic of skin morphology is superimposed over an image of cyokeratin immunofluorescence from a specimen of normal skin. The dashed line is drawn over the epidermal/dermal junction, with the basal cell layer sitting immediately superficial to this junction. (C) EGFR and IGF1R expression was quantified using CellProfiler by determining the percentage of cSCC cells expressing EGFR or IGF1R (* denotes $p < 0.05$ between normal skin and cSCC). A highly significant threefold increase in EGFR was seen in cSCC samples compared to

normal skin ($p=0.00077$), and a smaller but significant upregulation of IGF1R in cSCC samples was also observed ($p=0.041$).

Figure 2. Receptor phosphorylation response to small molecule inhibitors

(A) MET1 cells were harvested after incubation for 48 hours in the presence of either 500nM erlotinib or 150nM as indicated. Cell lysates were then immunoblotted for phospho-EGFR, total EGFR, phospho-IGF1R, and total IGF1R. While expression levels of the receptors did not change, the phosphorylated receptors were barely detectable by immunoblotting. Immunoblots for alpha-tubulin were used as loading controls. Similar results were observed in other cell lines (not shown). (B) Cell lines (MET1, MET4, SCC12, and SCC13) were incubated in either 500nM erlotinib or 150nM as indicated. Cell lysates were then used for phospho-EGFR-specific ELISA. The level of EGFR phosphorylation was normalized to the level observed in the control lysates for each cell line. Erlotinib treatment

significantly reduced EGFR phosphorylation in all cell lines (* denotes $p < 0.01$ relative to control), while PPP had no effect on EGFR phosphorylation. (C) Lysates from (b) were also used for phospho-IGF1R-specific ELISAs. In this case PPP treatment reduced IGF1R phosphorylation (* denotes $p < 0.05$ versus control) while erlotinib had no effect on IGF1R phosphorylation.

Figure 3. Erlotinib and PPP act together to reduce cell proliferation

MET1 cells were cultured for 48 hours in increasing concentrations of erlotinib (A) or PPP (B) as indicated. Cell growth was then assessed using MTT assays. A dose-dependent decrease in the number of viable cells is seen with rising inhibitor concentrations ($n=3$ at all time points). MET1 cells (C), MET4 cells (D), SCC12 cells (E), and SCC13 cells (F) were cultured for 48 hours in varying concentrations of erlotinib and PPP as indicated. Cell growth was then assayed with MTT assays. Both erlotinib and PPP demonstrated dose-dependent inhibition of cell growth, and at intermediate concentrations there appeared to be synergistic prevention of cell growth by both inhibitors. Higher concentrations of all

inhibitors were also tested but are not shown as they exceeded the maximal responses of the cells to the inhibitors.

Figure 4. Erlotinib and PPP prevent downstream signaling via Akt and p42/44 MAP kinase

(A) MET1 cells were harvested after incubation for 48 hours in the presence of either 500nM erlotinib or 150nM PPP as indicated. Cell lysates were then immunoblotted for phospho-Akt, total Akt, phospho-p42/44 MAPK, total p42/44 MAPK, phospho-mTOR, or total mTOR. α -tubulin immunoblots were performed as loading controls. While total protein levels are unchanged, there is a clear reduction in Akt and p42/44 phosphorylation in response to both erlotinib and PPP, although this is most pronounced in response to erlotinib. (B) Quantitation of several immunoblots for Akt shows a significant reduction in the relative amount of phosphorylated Akt versus total Akt after erlotinib treatment ($p=0.001$ vs control) and PPP treatment ($p=0.05$ vs control), which is even greater when both inhibitors are used in combination ($p=0.006$ vs control, 0.04 vs erlotinib only, 0.02 vs PPP only) (C) Similarly, quantitation of immunoblots for phospho-p42/44 reveals reduced phosphorylation after erlotinib treatment ($p=0.03$ vs control), PPP treatment ($p=0.05$ vs control), and both inhibitors in combination ($p=0.01$ vs control, 0.09 vs erlotinib only, 0.05 vs PPP only).

Table 1

IC50 values

Cell line	Erlotinib IC50 (nM)	PPP IC50(nM)
MET1	254	215
MET 4	268	143
SCC12	207	347
SCC13	357	186

Table 2

Results of MTT assays at intermediate concentrations

MET 1 cells	Absorbance	P value vs. combination therapy
No inhibitor	1.64±0.03	<0.001
+120nM erlotinib	1.44±0.07	0.004
+150nM PPP	1.36±0.06	0.061
+120nM erlotinib+150nM PPP	1.27±0.04	n/a
+500nM erlotinib	1.24±0.03	0.007
+150nM PPP	1.39±0.03	0.001
+500nM erlotinib+150nM PPP	1.09±0.02	n/a
MET4 cells		
No inhibitor	1.70±0.03	<0.001
+120nM erlotinib	1.52±0.02	<0.001
+120nM PPP	1.34±0.06	0.015
+120nM erlotinib+120nM PPP	1.17±0.06	n/a
SCC12 cells		
No inhibitor	1.02±0.02	0.001
+120nM erlotinib	0.94±0.03	0.030
+240nM PPP	0.95±0.01	0.003
+120nM erlotinib+240nM PPP	0.82±0.02	n/a
SCC13 cells		
No inhibitor	2.41±0.02	0.001
+120nM erlotinib	2.21±0.02	0.012
+60nM PPP	2.21±0.03	0.018
+120nM erlotinib+60nM PPP	2.00±0.04	n/a