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Original Article

EGFR inhibitors erlotinib and lapatinib ameliorate epidermal blistering in pemphigus vulgaris in a non-linear, V-shaped relationship

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Abstract: Novel insights into intra-cellular signalling involved in pemphigus vulgaris (PV), an autoimmune blistering disease of skin and mucous membranes, are now revealing new therapeutic approaches such as the chemical inhibition of PV-associated signals in conjunction with standard immunosuppressive therapy. However, extensive inhibition of signalling molecules that are required for normal tissue function and integrity may hamper this approach. Using a neonatal PV mouse model, we demonstrate that epidermal blistering can be prevented in a dose-dependent manner by clinically approved EGFR inhibitors erlotinib and lapatinib, but only up to approximately 50% of normal EGFR activity. At lower EGFR activity, blisters again aggravated and were highly exacerbated in mice with a conditional deletion of *EGFR*. Statistical analysis of the relation between EGFR activity and the extent of skin blistering revealed the best fit with a non-linear, V-shaped curve with a median break point at 52% EGFR activity ($P = 0.0005$). Moreover, lapatinib (a dual EGFR/ErbB2 inhibitor) but not erlotinib significantly reduced blistering in the oral cavity, suggesting that signalling mechanisms differ between PV predilection sites. Our results demonstrate that future clinical trials evaluating EGFR/ErbB2 inhibitors in PV patients must select treatment doses that retain a specific level of signal molecule activity. These findings may also be of relevance for cancer patients treated with EGFR inhibitors, for whom skin lesions due to extensive EGFR inhibition represent a major threat.

Key words: c-Myc – EGFR – EGFR inhibitors – erlotinib – lapatinib – p38MAK – pemphigus vulgaris signalling – pilot study

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Introduction

Pemphigus vulgaris is an autoimmune blistering disease of epidermis and mucous membranes. At disease onset, the majority of patients exhibit antibodies against the desmosomal cadherin desmoglein (Dsg) 3, leading to loss of intercellular adhesion in mucous membranes, in particular in the oral cavity (1,2). At a later point, following the appearance of anti-Dsg1 antibodies, blisters also spread to the epidermis. Over recent years, inadequate signal activation in antibody-targeted skin keratinocytes has been increasingly implicated in the pathophysiology of PV and is of particular interest for the development of novel treatment approaches in this often difficult to control disorder (3,4).

Morbidity due to immunosuppressive drug resistance as well as drug-induced side effects remains significant unresolved issues in the treatment of PV (5). Signal inhibition in target keratinocytes might therefore represent a promising first-line intervention to limit the spread of lesions. Proof of principle that such an approach is feasible has already been provided; however, discrepant results between different model systems currently jeopardize the translation of this promising approach into the clinic. For example, pretreatment with inhibitors dampening the activity of EGFR, p38MAPK, c-Myc, FAK or neural nitric oxide synthase prevented blistering in the epidermis of neonatal mice injected with PVIgG or the pathogenic, monospecific Dsg3 antibody AK23 (6–10). Intriguingly, epidermal blistering was, however, not abrogated in the epidermis of mice lacking the major p38MAPK isoform or in patients treated with p38MAPK inhibitors (11,12). In the latter case, clinical trials were also hindered due to hepatotoxicity of the compound (D. Rubenstein, pers. communication). Similarly, EGFR inhibition failed to prevent loss of intercellular adhesion in cultured normal human or HaCaT keratinocytes (13), while a first study combining PVIgG with the clinically approved EGFR inhibitor erlotinib reported on blister prevention in neonatal mice (7). Conflicting findings, as outlined above, casted doubts on the relevance of signalling effectors in PV pathophysiology and in particular on the feasibility of using inhibitors in a clinical setting.

In contrast to a previous report (7), a preliminary set of experiments revealed that higher doses of erlotinib than those used in the clinic (100 mg/kg versus 2.5 mg/kg body weight) failed to prevent epidermal blistering in a neonatal PV mouse model with confirmed EGFR activation upon treatment with PVIgG or AK23 in

combination with a half-maximal dose of pemphigus foliaceus (PF) IgG [to induce skin blisters (14)] (15,16). On the background of the knowledge that human patients with cancer develop skin rashes (17) and occasional bullous lesions (18) after prolonged treatment with erlotinib alone, our preliminary results suggested to us that too extensive EGFR inhibition might affect tissue integrity, which potentially exacerbates the antibody response and hampers the beneficial outcome of the inhibitor approach in PV. With the goal to provide the basis for a clinical pilot study on the use of inhibitors to signalling molecules in PV, we set out to address the hypothesis that the success of the inhibitor approach in PV is both defined and limited by the extent of EGFR inhibition.

Material and methods

Chemicals, antibodies and mice

Erlotinib (HCl salt, ChemieTek, CT-EL002) and lapatinib (ditosylate salt, ChemieTek CT-LP002) were dissolved in 100% DMSO diluted in PBS pH3 and administered i.p. at a final DMSO concentration of 12% and 19.6%, respectively. Four hours after application of inhibitors at indicated doses, AK23 [90 μg/g body weight; kind gift of Dr. Masayuki Amagai, Keio University, Tokyo (16)] together with a half-maximal dose of PF IgG [AK23/PF; PF1 (2.5 mg/g) or PF2 (1.25 mg/g)] or PVIgG (10 mg/g; Dsg3/Dsg1 antibodies) was subcutaneously injected in 2-day-old C57Bl/6J neonatal mice or conditionally deleted *EGFR* mice [$EGFR^{f/f}$ K5-Cre; (19)], as described previously (6,15). Control mice received the corresponding DMSO concentration in addition to mouse (Equitec) and/or human (Sandoglobuline) IgG. The following antibodies were used for Western blotting: P-Tyr (PY20; BD, 610000), EGFR (milipore, 06-847), c-Myc (Cell Signaling, 9402), P-p38MAPK (Thr180/Tyr182) (Cell Signaling, 9211) and tubulin (abcam, 6046). Experiments were approved by the ethics committee, Canton Bern, Switzerland [26/08 and BE78/11 (since 2012)].

Protein extraction, Western blot analyses and quantification of blisters

The entire skin was collected 4 h after inhibitor treatment or 24 h after antibody injection and processed for routine histology and protein extraction with lysis buffer containing 1% Triton X-100 followed by Western blot analyses, as described previously (15). Briefly, the top section of the skin was collected and subjected to protein extraction, while the remaining piece was cut on each side of the dorsal mid-line into 4–6 slices and paraffin embedded in the same cassette to evaluate the A and B half on the same slide (Figure S1c). Using Image J, epidermal and palate blisters were quantified by measuring on micrographs taken from serial cuts of all haematoxylin and eosin-stained slices. The extent of blisters is presented as % PV blisters calculated from the total blister length relative to the length of the entire biopsy.

Cultured keratinocytes and dissociation assay

C57BL\6J keratinocytes were cultured in CnT-02 medium (CELLnTEC, Switzerland), switched to 1.2 mM CaCl2 with or without indicated doses of erlotinib or DMSO, lysed 6 h later and processed for protein analyses as described (6). Alternatively, keratinocytes were treated with AK23 (20 μg/ml) and PF1 (280 μg/ml) for 48 h in presence or absence of indicated doses of erlotinib or DMSO and subjected to a dispase-based adhesion assay as described previously (20) (Figure S1d).

Statistical analyses

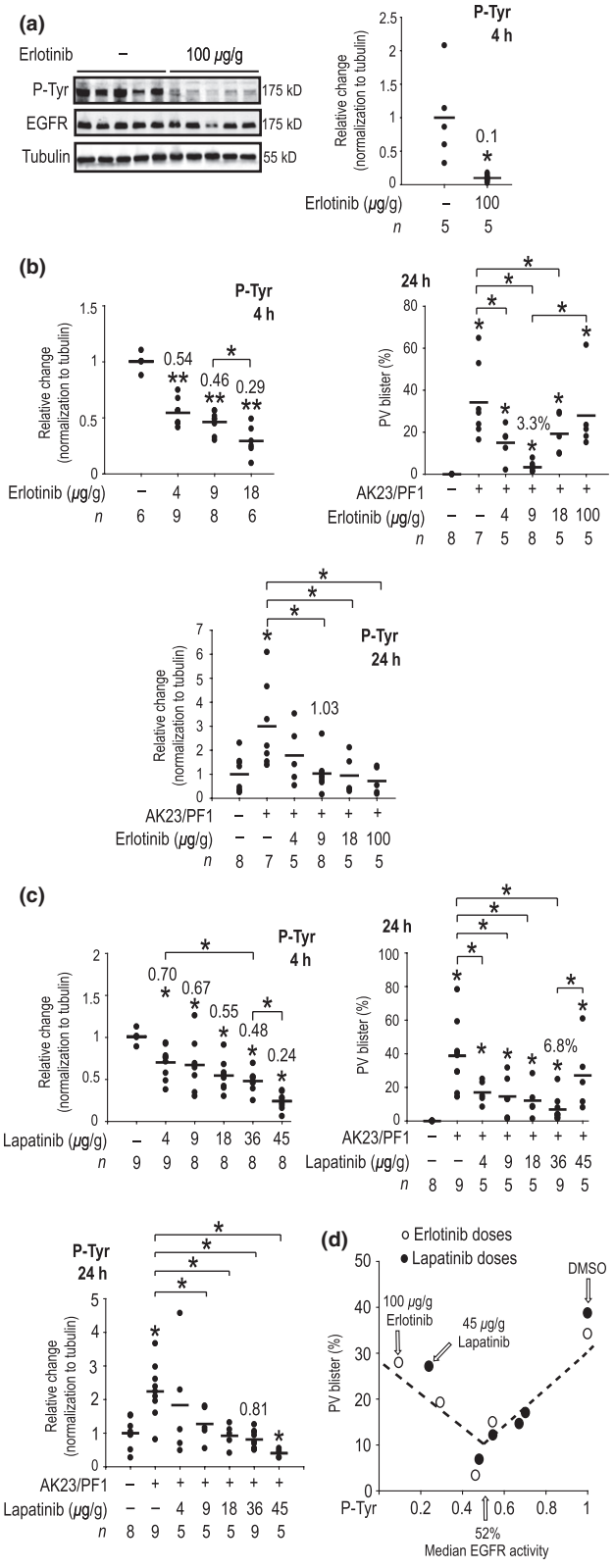
To assess relative changes, statistical analyses were performed using NCSS (Kaysville, UT). Significant differences between groups were defined using the nonparametric Kruskal–Wallis one-way ANOVA followed by Mann–Whitney *U*-test or directly by Mann–Whitney U-test with $P \leq 0.05$. The relationship between EGFR phosphorylation and skin blistering was investigated with a bootstrapping approach. Within one drug dosage, values were randomly paired to build 2000 data sets. For each data set, the fit of a linear relation was compared with a V-shaped (segmented) model by comparing the Akaike information criterion. The procedure was performed in R (version 2.15.0, the R Foundation for Statistical Computing) (21) using the functions lm and segmented (22,23). A *P*-value equivalent was computed as the proportion of differences greater or equal to zero. From the 2000 iterations, a median estimate for the break point (minimum) and the 95% confidence interval were generated.

Results

Erlotinib and lapatinib prevent epidermal blistering depending on residual EGFR activity

We initially observed that 100 μg/g body weight erlotinib, which was previously reported to prevent PV blisters (7), affected tissue integrity on its own and failed to reduce PV blisters in neonatal C57Bl\6J mice. Intra-peritoneal injection of this high-dose erlotinib alone resulted in focal sub- and intra-epidermal as well as suprabasal PV-like blisters in epidermis and/or palate of these mice 4 h after injection (Figure S1a). Pretreatment for 4 h further failed to reduce the blister size in the epidermis and palate of the neonatal C57Bl\6J mice injected with the pathogenic monoclonal antibody AK23 together with a half-maximal dose of PF IgG (AK23/PF) (15) (Table S1, Figure S1b). The extent of blistering was quantified by systematically measuring the blister lengths on sections obtained from the entire skin and palate (Figure S1c). Finally, we observed that the 100 μg/g body weight erlotinib pretreatment resulted in a drop of normal EGFR activity in the epidermis from 100% to 10% as measured by the level of tyrosine phosphorylation quantified by Western blotting of proteins extracted from the top piece of the analysed skin sections (Fig. 1a, Figure S1c).

We reasoned that the discrepancy with previous results using 100 μg/g erlotinib (7) could emanate from differences in solubility, that is, completely dissolved erlotinib in DMSO diluted in PBS pH3 (like used in clinics) in this study versus a suspension with potentially reduced solubility in the previous study (7). We therefore tested the efficacy of lower erlotinib concentrations to prevent blisters in a dose–response study between 4 and 18 μg/g body weight. At the time point of AK23/PF injection into C57Bl\6J neonatal mice, these doses less strikingly reduced tyrosine phosphorylation of EGFR than high-dose erlotinib, ranging in average from 54 to 29% of control levels (compare Fig. 1a, b, P-Tyr 4 h). Of these treatments, 9 μg/g erlotinib with 46% ($\pm$SD 9.9%) residual EGFR activity most efficiently inhibited blister formation, with on average only 3.3% blisters remaining (Fig. 1b). This beneficial effect was less pronounced with 4 μg/g and, in addition, was gradually lost with doses of erlotinib higher than 9 μg/g, conditions which more significantly reduced phosphotyrosine levels as compared to control (Fig. 1a, b, P-Tyr 4 h). In line



with former reports on EGFR activation in PV (7,15,24,25), EGFR phosphorylation after 24 h was increased on average by threefold in AK23/PF-injected animals (Fig. 1b, P-Tyr 24 h). At the same time point, EGFR phosphorylation was reduced to control levels in presence of 9 μg/g erlotinib.

The relationship between EGFR phosphorylation at the time point of AK23/PF injection and skin blistering was then modelled by a bootstrapping approach where values were randomly paired for one drug dosage to build 2000 data sets. A V-shaped (segmented) model performed significantly ($P = 0.0005$) better than a simple linear model, indicating that there is a minimal break point in the relation of blistering and EGFR activity.

We then tested lapatinib, which also induces skin rashes (26) and is used in clinics at the highest daily dose of 1600 mg (on average 23 mg/kg body weight). While erlotinib predominantly inhibits EGFR but not ErbB2 (27), lapatinib is a dual EGFR and ErbB2 inhibitor (28). Four hours after lapatinib administration to neonatal C57Bl\6J mice between 4 and 45 μg/g body weight, EGFR activity was on average reduced to the same range as observed for erlotinib (compare Fig. 1b, c, P-Tyr 4 h). Furthermore, 36 μg/g efficiently diminished blister size, on average from 38% to 6.8% while EGFR activity was around control level after 24 h (Fig. 1c). In compliance with the supplier, reduced compound solubility did not allow the analyses of higher doses than 45 μg/g, providing limited data beyond the minimal EGFR activity to support a second, rising segment and thus no significant evidence for a V-shaped relationship ($P = 0.085$). However, the joint data set of erlotinib and lapatinib supported a segmented relationship ($P = 0.0005$) with minimal blistering at the median EGFR activity of 52% (95% confidence interval: 36–69%) (Fig 1d).

Together these results indicate that both erlotinib and lapatinib efficiently prevent blister formation in the epidermis of AK23/PF-injected mice, supporting the involvement of EGFR in this process. Furthermore, the inhibitor response in terms of blistering followed a V-shaped curve, predicting that these inhibitors most efficiently prevent blistering when operating in a window between 36 and 69% residual EGFR activity.

Erlotinib and lapatinib equally prevent AK23/PF and PVIgG-induced epidermal blisters

We further tested the efficacy of erlotinib and lapatinib to prevent PVIgG (containing Dsg3 and Dsg1 antibodies)-induced blistering. In presence of the most efficient erlotinib and lapatinib doses of 9 and 36 μg/g body weight, respectively, PVIgG-induced blistering was prevented to a comparable extent as for AK23/PF (Fig. 2). Furthermore, no blisters were observed with inhibitors alone. After

Figure 1. EGFR activity and blister formation in the epidermis of AK23/PF1-injected C57Bl\6J neonatal mice pretreated for 4 h with erlotinib or lapatinib. (a) Western blot analysis with indicated antibodies 4 h after erlotinib injection and graph showing Odyssey (Licor) quantification of phosphotyrosine signals at the level of EGFR normalized to tublin. Data are presented relative to the average of control animals injected with vehicle (DMSO and corresponding control IgG) set to 1 in each litter. (b) Erlotinib dose–response study for indicated dosages; quantification of phosphotyrosine signals on Western blot as indicated in a, 4 h after erlotinib injection and 24 h after AK23/PF treatment, respectively. The size of PV blister is indicated at 24 h as % of the entire biopsy. (c) As in b, except that lapatinib was used as an EGFR/ErbB2 inhibitor. (d) Blistering versus phosphotyrosine levels; for each dosage of erlotinib and lapatinib, the mean of the blistering and the phosphotyrosine level is plotted. The dashed line depicts the mean V-shaped relation calculated with the bootstrapping procedure. *n*; number of animals per group; a–c: horizontal bars represent the mean; $*P \leq 0.05$, relative to DMSO control, unless indicated otherwise; d: dots represent means.

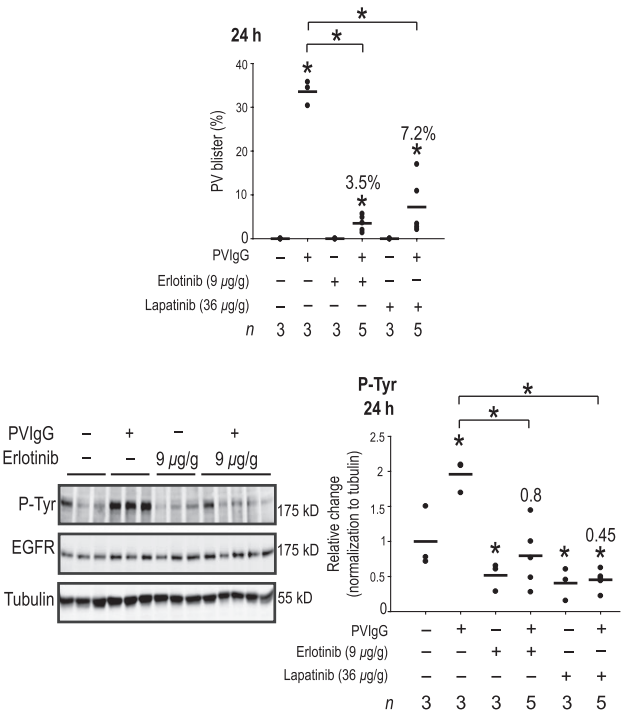


Figure 2. PVIgG-injected C57Bl\6J neonatal mice pretreated with Erlotinib or Lapatinib for 4 h. The size of PV blisters is indicated at 24 h as % of the entire biopsy in neonatal mice treated with erlotinib or lapatinib. Lower panel: Western blot analyses show indicated antibodies 24 h after inhibitor injection, and graph depicts quantification of phosphotyrosine signals on Western blot at 24 h. *n*; number of animals per group; horizontal bars represent the mean; data are presented relative to the average of control animals injected with vehicle set to 1; $*P \leq 0.05$, relative to DMSO control, unless indicated otherwise.

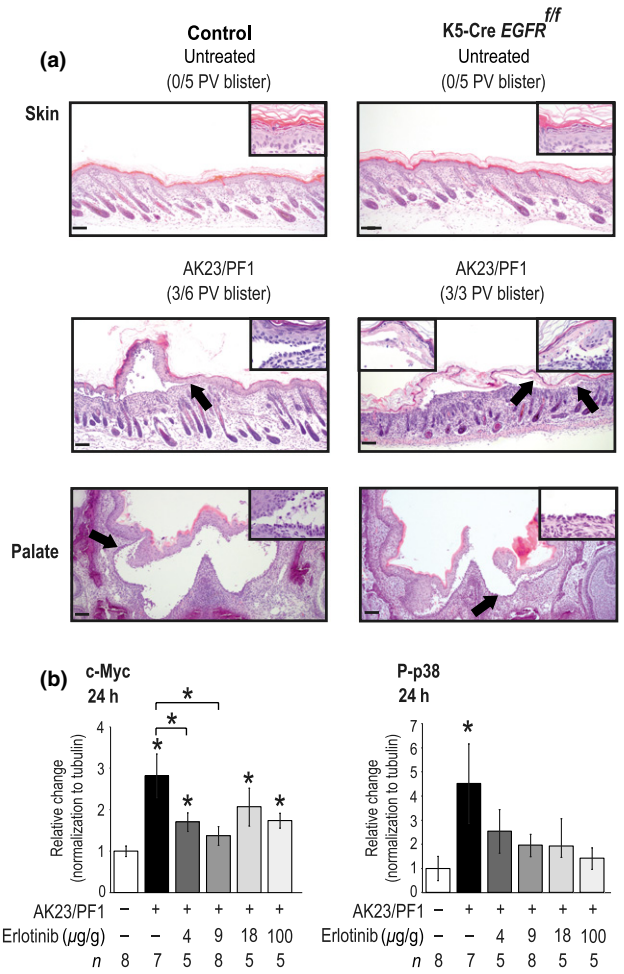


Figure 3. Blister formation in AK23/PF1-injected *K5-Cre-EGFRf/f* mice genetically deleted for *EGFR* and signal activation in AK23/PF1-injected C57Bl\6 neonatal mice. (a) Representative histomicrographs of skin and palate from neonatal control and conditionally deleted *EGFR* littermates [$EGFR^{f/f}$ K5-Cre; (19)] without (top panel) or with treatment (*n [control]* = 5, *[control, AK23/PF1]* = 6; [$EGFR^{f/f}$ *K5-Cre*] = 5, [$EGFR^{f/f}$ *K5-Cre; AK23/PF1*] = 3). The number of animals exhibiting blisters is indicated between parentheses. Inserts are 10 × magnification of selected areas indicated by arrows. Note loss of tissue integrity in the blister roof of *EGFR*-deleted epidermis (left insert). Scale bars = 100 μm. (b) Graphs show Odyssey (Licor) quantification from Western blot analysis of skin protein extracts from animals from Figure 1 with indicated antibodies 24 h after erlotinib injection. Data were normalized to tublin and are presented relative to the average of control animals injected with vehicle in each litter set to 1. $*P \leq 0.05$, relative to DMSO control.

24 h, phosphotyrosine levels were still below control (Fig. 2, P-Tyr 24 h) but within the calculated range of residual EGFR activity.

Extensive EGFR inhibition enhances antibody responsiveness of cultured keratinocytes

Our results suggested that the inhibitor response in terms of blistering follows a V-shaped rather than a linear dose–response curve. To confirm that this effect was mediated by the keratinocyte itself, we pre-incubated epidermal mouse keratinocyte cultures with increasing erlotinib concentrations. We analysed phosphotyrosine levels of EGFR prior to AK23/PF treatment as well as alterations of the cell's adhesive strength according to a previously established dissociation assay that involves application of mechanical stress (20). Phosphotyrosine levels were on average higher than *in vivo* before antibody treatment but the response profile after 48 h in terms of loss on intercellular adhesion also clearly followed a V-shaped pattern; erlotinib best prevented loss of cell–cell adhesion at 0.4 μg/ml while disruption of the cellular sheet gradually increased with higher inhibitor doses and lower EGFR activity (Figure S1d).

Loss of EGFR function is critical in presence of Dsg3/Dsg1 antibodies

If the level of EGFR activity is critical in the presence of Dsg3/Dsg1 antibodies, complete loss of EGFR was expected to exacerbate the blistering phenotype. This question was addressed using transgenic neonatal mice with a conditional *EGFR* deletion in epidermis and mucous membranes (19). *EGFR* deletion alone did not result in blistering in these mice 2 days after birth (Fig. 3a). However, in comparison with injected control mice, AK23/PF greatly enhanced the epidermal blistering phenotype of all *EGFR*-deleted neonatal mice analysed and was further associated with poor survival (3/6 animals); besides typical suprabasal PV blister affecting over 95% of the entire skin, adhesion between keratinocytes and tissue integrity in the blister roof was also lost. The latter was not seen with high doses of Erlotinib and AK23/PF (Table S1) and might be related to phenotypic barrier defects and inflammation becoming apparent in *EGFR*-deleted mice shortly after birth (29). In contrast to the epidermis, the extent of AK23/PF-induced blistering in the palate was comparable between control mice and mice deleted for *EGFR*, raising the possibility that EGFR

does not play the same role for tissue integrity in epidermis and oral mucosa and that signalling pathways involved in blister formation might differ between these two sites. Performing PV IgG injection into *EGFR*-deleted mice would have been advantageous, proved, however, difficult for ethical reasons due to the vast extent of blistering associated with poor survival in response to Dsg3/Dsg1 antibodies.

The *EGFR* deletion and EGFR inhibitor experiments underscored that extensive EGFR inhibition exacerbated the effect of Dsg3/Dsg1 antibodies. As this phenomenon was seen after short-term treatment with inhibitors precluding major structural changes, we reasoned that it could be mediated through enhanced activation of signalling effectors normally contributing to PV. We investigated p38MAPK and c-Myc in the presence of AK23/PF and erlotinib. These effectors can be regulated by EGFR and contribute to PV blistering (6,8) (Fig. 3b). The steady-state levels of activated p38MAPK (P-p38MAPK) at 24 h decreased in a dose-dependent manner and paralleled the EGFR inhibition profile for erlotinib (Fig. 3b compare to Fig. 1b). However, c-Myc levels increased after the break point, reflecting the segmented blistering profile.

Lapatinib but not erlotinib significantly prevents AK23/PF- and PVIgG-induced oral blisters

Due to differential findings in epidermis and oral cavity of mice with deleted *EGFR* (Fig. 3a), we analysed oral blisters in the same mice that had served to study epidermal blistering after erlotinib and lapatinib treatment (Figs 1 and 2). As suggested by *EGFR* gene deletion, none of the erlotinib (inhibiting EGFR) concentrations significantly reduced AK23/PF-induced blistering in the palate (Fig. 4a). However, lapatinib (inhibiting EGFR and ErbB2) prevented blistering to a similar extent than in the epidermis following a V-shaped dose–response curve (Fig. 4b). Furthermore, lapatinib treatment also significantly reduced PVIgG-induced blistering. Together, these results support the possibility that in PV, the relative contribution of EGFR family members to blister formation in the oral cavity differs from the epidermis (Fig. 4c).

Discussion

Our data demonstrate that PV blistering can be prevented by inhibition of EGFR in the epidermis or EGFR/ErbB2 in the oral cavity using erlotinib/lapatinib or lapatinib, respectively. However, an equally important finding is that the beneficial effect is only observed when EGFR in the epidermis is inhibited in the range between 36 and 69% of its normal activity while the benefit is gradually lost at higher inhibition following a non-linear, V-shaped dose–response curve.

The V-shaped instead of a linear dose response is a novel finding for PV inhibitor treatments, strengthened by the observation that it occurs with two different inhibitors, was recapitulated *in vitro* and is acerbated upon *EGFR* deletion. Consistent with our working hypothesis, these data further support that the increase in blistering after the break point relates to the activity of EGFR itself rather than to toxicity of the inhibitors. In other terms, this phenomenon appears to be an 'on-target' rather than 'off-target' effect of the inhibitors.

One plausible explanation for increased Dsg3/Dsg1 antibody-induced blistering in combination with reduced EGFR activity consists in the comparable activation of blister-triggering signalling effectors in the V-shaped curve on both sides of the break point. An example for such an effector is c-Myc, which has been involved in the blistering process (6) and was found in this study to be above control level, both with too low and too high doses of erlotinib.

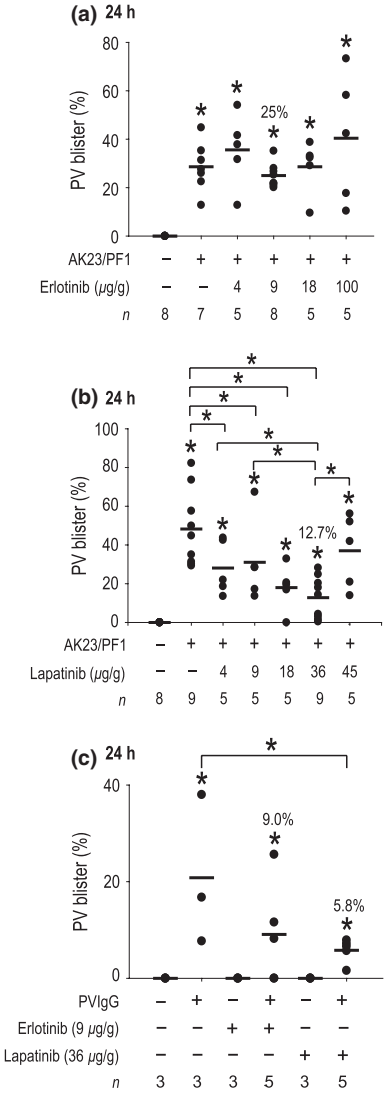


Figure 4. Blister quantification in the palate of AK23/PF1- or PVIgG-injected C57Bl\6J neonatal mice pretreated for 4 h with erlotinib or lapatinib. (a, b, c) Quantification of PV blisters indicated as % of the entire biopsy for indicated treatments.

Observations concerning the role of classical cadherins in differentiation and EGFR signalling may provide an explanation for the upregulation of c-Myc by both high and low EGFR activity. In mouse keratinocytes, the signal transduction by E-cadherin under normal homeostatic conditions was found to be dependent on both E-cadherin adhesion and a certain EGFR activity (30). As E-cadherin interacts with EGFR but also with Dsg3 (31,32), EGFR inhibition below a critical threshold may affect Dsg3 signalling to a similar extent than reported for E-cadherin. PV antibody binding also disrupts Dsg3 signalling resulting in c-Myc upregulation (6). Too extensive EGFR inhibition could therefore exacerbate the antibody effect resulting in an amplification loop involving

c-Myc. In contrast to c-Myc, increasing erlotinib concentrations decreased P-p38MAPK in a linear dose-dependent manner not exceeding base levels. This supports that at the time point of our analysis, p38MAPK is directly dependent on EGFR signalling but is most likely not involved in increased blistering in combination with reduced EGFR activity after the brake point. However, the elucidation of this phenomenon requires a better understanding of the complex signalling network downstream of EGFR and Dsg3 with and without antibodies, an undertaking which is beyond the scope of the current study. The finding that the signalling status of the keratinocyte critically impacts on blister formation, underscored by these findings, provides, however, a rational for the clinical observation that keratinocytes of a specific differentiation stage, such as in the basal layer, are responsive to PV antibodies, while suprabasal cells are not.

In summary, our results show for the first time that (i) blistering in PV can be ameliorated by partial inhibition of EGFR, whereas inhibition above or below this level reverts this condition, and that (ii) blistering in the epidermis but to a lesser extent in the palate appears to primarily depend on loss of desmosomal cadherin function combined with EGFR activity. These data have two key clinical implications; firstly, treatment of PV-induced blistering with EGFR inhibitors will require careful titration of the inhibitor dose to hit the effective treatment window, and secondly, the dual-nature of lapatinib inhibition is likely to be more favourable than erlotinib for combined treatment of blisters in epidermis and palate. Finally, it is conceivable that skin rashes in patients with cancer treated with EGFR inhibitors may also be controlled by an inhibitor dose carefully selected to preserve sufficient EGFR activity for tissue integrity.

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Author Contributions

B.S. performed the research, with exception of the experiment cited below. She was helped by M.Siffert (animal keeper) and supervised by E.J.M. and A.G. She also designed the experiments under the supervision of E.J.M. and A.G. S.R. performed the statistical analyses. E. S. provided the PF an PVIgG and M. S. the EGFR-deleted mice as well as lab space to perform the experiments, which were performed by AG. B.S. provided the first draft of the manuscript, written by E.J.M. together with all co-authors.

Conflict of interests

The authors have declared no conflicting interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Skin biopsies and *in vitro* studies.

Table S1. Overview of high dose Erlotinib treatment (100 μg/g body weight) which fails to significantly alter blister size.