



# Inhibition of poxvirus spreading by the anti-tumor drug Gefitinib (Iressa<sup>TM</sup>)

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## ABSTRACT

The threat of smallpox virus as a bioterrorist weapon is raising international concerns again since the anthrax attacks in the USA in 2001. The medical readiness of treating patients suffering from such infections is a prerequisite of an effective civil defense system. Currently the only therapeutic option for the treatment of poxvirus infections relies on the virostatic nucleoside analog cidofovir, although severe side effects and drug resistant strains have been described. A growing understanding of poxvirus pathogenesis raises the possibility to explore other appropriate targets involved in the viral replication cycle. Poxvirus encoded growth factors such as the Vaccinia Growth Factor (VGF) stimulate host cells via the Epidermal Growth Factor Receptor (EGFR) and thereby facilitate viral spreading. In this study we could visualize for the first time the paracrine priming of uninfected cells for subsequent infection by orthopoxviruses directly linked to EGFR phosphorylation. Since EGFR is a well known target for anti-tumor therapy small molecules for inhibition of its tyrosine kinase (TK) activity are readily available and clinically evaluated. In this study we analyzed three different EGFR receptor tyrosine kinase inhibitors for inhibition of orthopoxvirus infection in epithelial cells. The inhibitor shown to be most effective was Gefitinib (Iressa<sup>TM</sup>) which is already approved as a drug for anti-tumor medication in the USA and in Europe. Thus Gefitinib may provide a new therapeutic option for single or combination therapy of acute poxvirus infections, acting on a cellular target and thus reducing the risk of viral resistance to treatment.

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## 1. Introduction

In 1980, the WHO reported the worldwide eradication of Smallpox as a result of a global vaccination initiative with Vaccinia virus (WHO, 1980). Protection against smallpox virus infections seemed no longer necessary and vaccination programs were stopped also due to an unfavourable risk–benefit profile (Fenner et al., 1988). Consequently the population of unvaccinated individuals is growing steadily until today. Since several years and in particular after the anthrax attacks in the US in 2001 increasing concerns about the use of smallpox virus as a bioterrorist weapon reinforce the importance of a clinical response against poxvirus infections (Jahriling et al., 2005; Tegnell et al., 2002).

The only therapeutic option for the treatment of poxvirus infections currently relies on the nucleoside analog cidofovir, but drug-resistant strains have already been described and therapy is dose-limited by nephrotoxic side effects (Topalis et al., 2005; Smee et al., 2002; Ortiz et al., 2005). Although other promising compounds for the treatment of poxvirus infections are under

development they are not yet completely clinically evaluated and approved by the health authorities yet (Jordan et al., 2008; Smith et al., 2009).

A growing understanding of poxvirus replication and the mechanisms that lead to the pathogenesis of poxvirus infections raises the possibility to find new targets for the treatment of such infections. Poxviruses encode growth factor proteins that facilitate the viral replication in host cells by signaling through cellular growth factor receptors belonging to the tyrosine kinase family (Tzahar et al., 1998). The Vaccinia Growth Factor (VGF) corresponds to the Smallpox Growth Factor (SPGF) encoded by Variola virus (Kim et al., 2004). Vaccinia virus and Variola virus both belong to the highly homologous and genetically and immunologically closely related family of orthopoxviruses (Fenner et al., 1988). The secreted epidermal growth factor (EGF)-like protein VGF binds and signals through the EGF-receptor (EGFR, ErbB1) (Brown et al., 1985). EGFR belongs to the ErbB1–4 group of receptor tyrosine kinases that form homo- and heterodimers upon ligand binding and activate the intracellular pathways Ras-Mek-ERK, phospholipase C and STAT (Yarden, 2001; Silva et al., 2006). VGF is an early viral protein that enhances the viral pathogenesis (Buller et al., 1988a,b; Opgenorth et al., 1983), stimulates cell proliferation and leads to the inhibition of apoptosis through EGFR signaling (Jost et al., 2001a,b; Postigo et al., 2009).

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Name	PD 153035	Vandetanib ZD6474, Zactima	Gefitinib Iressa
Mechanism	EGFR tyrosine kinase inhibitor	EGFR / VEGFR tyrosine kinase inhibitor	EGFR tyrosine kinase inhibitor
Molecular Formula			
IC50 Values	$\leq 1\mu\text{M}$ (Bos et al., 1997)	3.5 $\mu\text{M}$ - 80 $\mu\text{M}$ (Azzariti et al., 2006)	7.3 $\mu\text{M}$ - >100 $\mu\text{M}$ (Azzariti et al., 2006)
Clinical Phase	Experimental compound	Completed phase III trials in different cancer indications (Scagliotti and Govindan, 2010)	FDA / EMEA approved for NSCLC (Sequist et al., 2008; Cohen et al., 2003)

**Fig. 1.** Overview of EGFR tyrosine kinase inhibitors. Presented are the structural formula, IC<sub>50</sub> ranges from selected cell based assays as an example, and the current phase of clinical evaluation.

Most of the biological characteristics of EGFR and its role in pathogenesis are known from cancer research. EGFR signaling plays a key role in tumorigenesis and thus is a major target for anti-tumor therapy with monoclonal antibodies and small molecule inhibitors of tyrosine kinase activity (Okamoto, 2010).

In 2004, Kim et al. showed a significant efficacy of blocking EGFR tyrosine activity for antiviral treatment against orthopoxvirus infections by applying SPGF specific monoclonal antibodies in a murine Vaccinia pneumonia model (Kim et al., 2004). In 2005, Yang et al. analyzed the inhibition of EGFR signaling for the treatment of orthopoxvirus infections. Using the experimental tyrosine kinase inhibitor CI-1033 (Canertinib) they demonstrated a blocking of secondary viral spread of Variola virus *in vitro* and a strong antiviral activity in a Vaccinia virus mouse model (Yang et al., 2005). Currently CI-1033 is in clinical phase II studies for the treatment of different tumor types (Ocana and Amir, 2009).

Among the very few compounds clinically evaluated and approved by the FDA for use as a therapeutic drug inhibiting EGFR signaling in cancer patients is Gefitinib. Gefitinib is an orally available small molecule tyrosine kinase inhibitor (TKI) (Harandi et al., 2009). It is marketed under the product name Iressa<sup>TM</sup> for the treatment of patients with Non Small Cell Lung Cancer (NSCLC) with activating mutations of the EGFR tyrosine kinase (Sequist et al., 2008).

This study is intended to determine the efficacy of three different EGFR tyrosine kinase inhibitors, PD153035, Vandetanib and Gefitinib, for the treatment of poxvirus infections. Since Gefitinib, is already approved as an anti-tumor drug our data might provide a new therapeutic option for the treatment of acute poxvirus infections by applying a clinical therapy protocol for Iressa<sup>TM</sup> (Fig. 1).

## 2. Materials and methods

### 2.1. Receptor tyrosine kinase inhibitors (RTKIs) small molecules

PD153035, Vandetanib and Gefitinib were obtained from SelleckChem (#S1079, #S1046, #S1025), and solved in DMSO for preparing stock solutions. Working concentrations were prepared

by further dilution in Dulbecco's modified Eagle's medium (DMEM, Gibco) without supplements.

### 2.2. Virus and cells

Vaccinia virus (VACV) Western Reserve (ATCC, #VR-1354) and cowpox virus (CPXV) Brighton (ATCC, #VR-302) were propagated in Hep2 cells (ATCC, #CCL-23) in Dulbecco's modified Eagle's medium (DMEM) (Gibco) with 5% fetal calf serum and 1% glutamine at 37 °C in a 5% CO<sub>2</sub> humidified atmosphere.

For the preparation of virus stocks infected cells were incubated for approximately four days until a pronounced cytopathic effect was observed. Supernatant was harvested from infected cell cultures centrifuged for 10 min at 200 × g to pellet cell debris. The infectivity titers of VACV and CPXV stocks were determined by a plaque assay on Hep2 cells (Kramski et al., 2010). Virus titers were expressed as plaque forming units (pfu) per mL. Virus suspensions were prepared by dilution of virus stock in DMEM without supplements.

### 2.3. Plaque reduction test

8 × 10<sup>4</sup> Hep2 cells were seeded 24 h before infection in 48 well tissue culture plates (Nunc) in 400 μl serum reduced growth medium (DMEM, 1% fetal calf serum, 1% glutamine). Medium was aspirated and 100 μl of RTKI compounds were added to the cells in concentrations as indicated in quadruplicates. After incubation for 1 h at 37 °C and 5% CO<sub>2</sub>, 100 μl VACV or CPXV suspension corresponding to 6.25 pfu/well or 12.5 pfu/well was added to the cells. Virus was allowed to adsorb for 3 h at 37 °C and 5% CO<sub>2</sub>. Cells were overlaid with 200 μl of 1.6% CMC and incubated for further 96 h at 37 °C and 5% CO<sub>2</sub>. Medium was aspirated and fixation and staining of cells were done identically to the plaque assay (Kramski et al., 2010). In some cases a proliferation assay was performed before the medium was aspirated. The number of plaques/well was counted and the plaque area versus the total area of one well was calculated in percent using imaging software ImageJ (NIH, USA).

## 2.4. Immunoblotting

Cells cultured as indicated for plaque reduction tests were lysed with RIPA buffer 1× (Thermo Scientific) supplemented with Halt phosphatase & proteinase inhibitor cocktail (Thermo Scientific). Proteins were fractionated on 8% SDS-PAGE gels and semi-dry blotted for 30 min at 200 mA. Primary antibodies anti-pEGFR phospho Y1068 (Abcam, #40815-100, 1/2000), anti-ERK1/2 (Cell Signaling, #4695, 1/2000), anti-pERK1/2 Thr202/Tyr204 (Cell Signaling, #4370, 1/2000), anti-VACV Lister Strain (Acris, #BP1076, 1/2000), anti-β-actin (Cell Signaling, #49672, 1/3000) were applied in PBS containing 3% milk powder and 0.1% Tween 20 (Sigma Aldrich). A peroxidase conjugated goat anti-rabbit IgG (Cell Signaling, #7074, 1/3000) was used as secondary antibody. Detection was performed by using Pierce ECL Western blotting substrate (Thermo Scientific).

## 2.5. Real-time PCR assay

$8 \times 10^4$  Hep2 cells were seeded 24 h before infection in 400 μl volume in 48 well tissue culture plates (Nunc) in serum reduced growth medium (DMEM, 1% fetal calf serum, 1% glutamine). Medium was aspirated and RTKI compounds in indicated molarities were added in 100 μl volume to the cells in quadruplicates. After incubation for 1 h at 37°C and 5% CO<sub>2</sub>, 100 μl VACV or CPXV suspension in 100 μl volume corresponding to 6.25 pfu was added to the cells. Cells were further incubated for 24 h at 37°C and 5% CO<sub>2</sub>. DNA was extracted from cells according to standard procedures using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). An orthopoxvirus specific real-time PCR was carried out as described (Schroeder and Nitsche, 2010) and genome equivalents of orthopoxviruses were calculated by using an orthopoxvirus plasmid standard.

## 2.6. Proliferation assay (Alamar Blue)

Reduction of the redox dye resazurin (Alamar Blue) to resorufin (O'Brien et al., 2000) by mitochondrial, cytosolic, and microsomal enzymes (Gonzalez and Tarloff, 2001) was used to measure possible cytotoxic effects of RTKI compounds in plaque assays. After incubation as described for the plaque reduction test Alamar Blue was added in a ratio of 1/20 to the cell culture medium and resorufin formation was measured spectrophotometrically (535/590 nm, using a Tecan Infinite plate reader).

## 2.7. Immunofluorescence

Immunostaining was performed on formalin-fixed cells incubated as described for the plaque reduction test on chamber slides. Cells were permeabilized with PBS 0.2% Triton x-100. Antigens were stained with antibodies against pEGFR (Abcam, #40815-100, phospho Y1068, 1/250) and human Vaccinia Immunoglobulins (VIG) (BEI Resources, #6804253, NR-2632, 1/250). Antigen-antibody complexes were detected with the appropriate secondary antibodies: FITC conjugated anti-human IgG (Invitrogen, #H10001C, 1/250), Cy3 conjugated anti-rabbit IgG (Dianova, #711-165-152, 1/250). Unspecific binding sites were blocked with PBS 0.1% Tween 20 and antibodies were diluted in PBS 0.1% Tween 20. Nuclei were counterstained with DAPI reagent (Serva, #18860, 1/50).

## 2.8. Statistical analysis

Student's *t*-test was performed with MSExcel (Microsoft Office). IC50 calculation for Gefitinib was carried out by a nonlinear regression fit curve analysis using Graph PadPrism 5.

## 3. Results

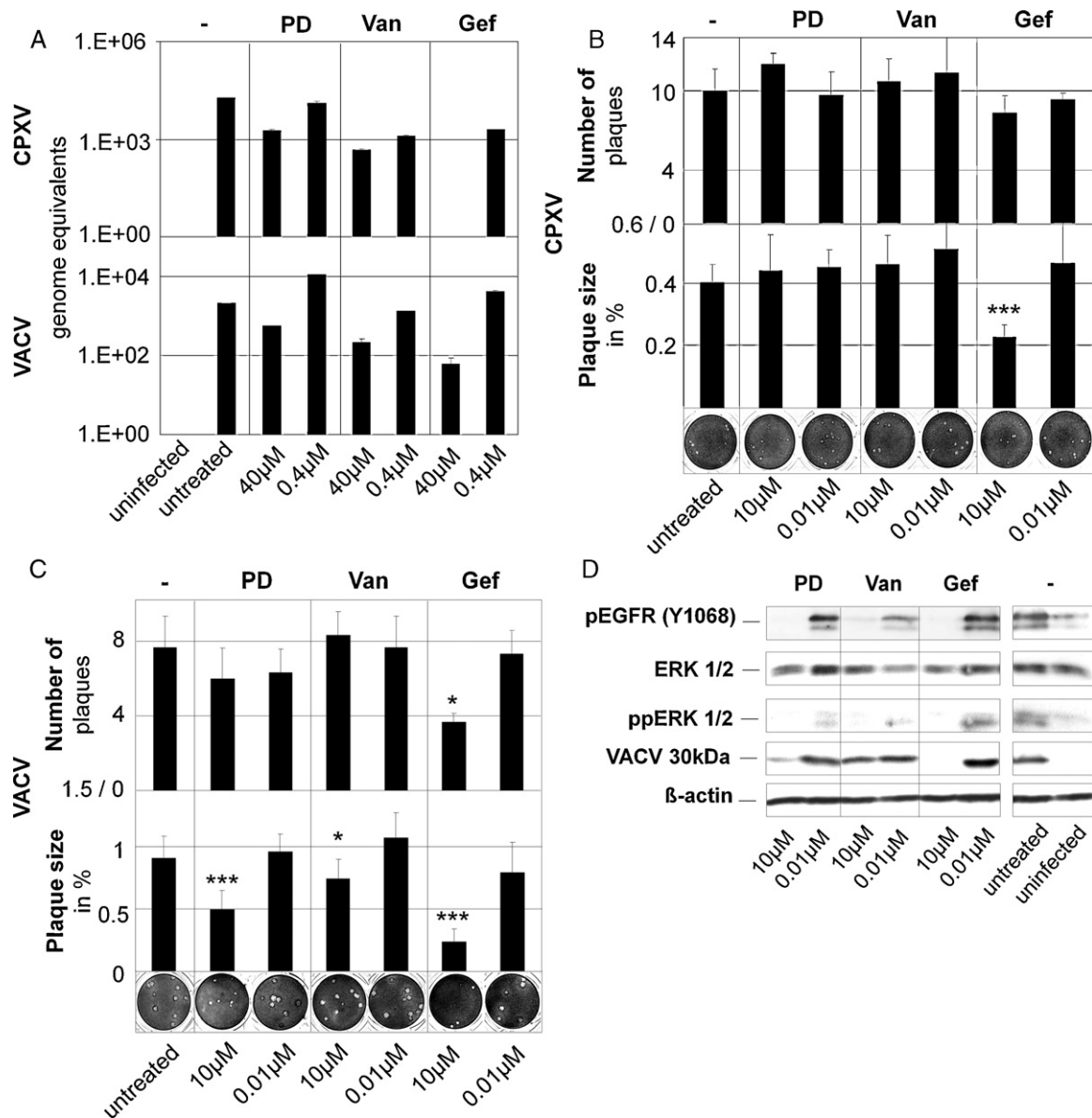
### 3.1. Treatment with EGFR inhibitors significantly reduces orthopoxvirus DNA replication

To examine the effect of the EGFR tyrosine kinase inhibitors PD153035, Vandetanib and Gefitinib on the infection of epithelial cells by VACV and CPXV, Hep2 cells were pre-treated with the compounds and subsequently infected with 12.5 pfu of VACV or CPXV. Based on the results of preliminary experiments (data not shown) the inhibitors were chosen to be tested at equimolar concentrations of 40 μM and 0.4 μM compared to infected, untreated and uninfected, untreated cells. The chosen molarities also cover the ranges of selected IC50 values for EGFR inhibitors reported for other cell based assays (Bos et al., 1997; Azzariti et al., 2006; Fig. 1). 24 h after infection viral genome equivalents were determined in a quantitative orthopoxvirus specific real-time PCR assay in Hep2 cell lysates. A significant reduction of orthopoxvirus DNA was observed in cells infected with VACV or CPXV treated with compounds at concentrations of 40 μM compared to untreated cells. This effect was reduced or not present at compound concentrations of 0.4 μM. The most effective inhibitor of VACV and CPXV DNA replication was Gefitinib. In Hep2 cells infected with CPXV and treated with 40 μM Gefitinib the detection of viral DNA was below the detection limit of 10 orthopoxvirus genome equivalents. The same treatment of cells infected with VACV lead to the detection of less than 100 (62) orthopoxvirus genome equivalents. In contrast in infected, untreated cells clearly higher copy numbers of genome equivalents were found for VACV (1998) and CPXV (18835) (Fig. 2A).

### 3.2. Treatment significantly inhibits orthopoxvirus spreading and correlates with impaired EGFR signaling

It was shown previously that treatment with an EGFR inhibitor leads to reduced size but not to significant reduced numbers of viral plaques in orthopoxvirus infected monolayer cell cultures (Yang et al., 2005). To analyze if such an effect could also be observed for PD153035, Vandetanib and Gefitinib, a plaque reduction test was performed with VACV and CPXV in Hep2 cells. The plaque reduction tests were analyzed for plaque numbers as well as for plaque sizes compared to the total well area size by imaging software ImageJ (Fig. 2B and C). The most prominent effect seen in the plaque reduction tests was significant reduction in plaque sizes by all compounds at concentrations of 10 μM in cells infected with VACV. In cells infected with CPXV Gefitinib was the only compound reducing plaque sizes significantly, while PD153035 and Vandetanib did not. Also Gefitinib was the only compound that was able to reduce the plaque number in VACV infected cells at a concentration of 10 μM significantly ( $p < 0.001$ ). No such effect was observed for PD153035 or Vandetanib in VACV or CPXV infected cells.

For a better understanding of the molecular mechanism involved in plaque size reduction by EGFR inhibitors we analyzed the phosphorylation status of EGFR, the downstream signaling kinases ERK1/2 as well as orthopoxvirus proteins in cell culture lysates of the VACV plaque reduction tests by Western blotting (Fig. 2D). Firstly we found a difference between the status of EGFR signaling in infected and uninfected Hep2 cells. The amount of phosphorylated EGFR and ERK1/2 was significantly increased in VACV infected cells. This increase of the activated kinases, pEGFR and pERK1/2, was abolished by the treatment with EGFR inhibitors PD153035, Vandetanib and Gefitinib at concentrations of 10 μM. Treatment of VACV infected Hep2 cells with inhibitor concentrations of 200 nM showed a superior inhibition of EGFR phosphorylation by Gefitinib (data not shown). Thus the inhibition of virus spreading by EGFR inhibitors observed in plaque reduction tests correlates with an impaired phosphorylation of EGFR



**Fig. 2.** Inhibition of orthopoxvirus replication and spreading correlates with blocking of EGFR signaling by PD153035 (PD), Vandetanib (Van) and Gefitinib (Gef). (A) Orthopoxvirus genome equivalents analyzed by real-time PCR of CPXV and VACV infected (6.25 pfu/well) and treated (40 μM or 0.4 μM) or untreated Hep2 cells. Uninfected, untreated and infected, untreated cells serve as controls. (B and C) Results from plaque reduction tests of Hep2 cells infected with CPXV (B) or VACV (C), treated with inhibitors (10 μM or 0.1 μM) and analyzed for plaque numbers and for plaque size in percent. Statistical significance was calculated by Student's *t*-test (\**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.005). (D) Western blot of cell lysates obtained from VACV plaque reduction tests for analysis of EGFR-ERK1/2 signaling and VACV proteins. As an indicative band for VACV a 30kDa protein was depicted from detection of total VACV proteins by a polyclonal antibody in cell lysates of infected Hep2 cells.

and ERK1/2 and with a reduced amount of VACV proteins. In line with the plaque reduction tests Gefitinib treatment of infected cells resulted in the strongest reduction of VACV proteins in Western blot analysis compared to PD153035 and Vandetanib at inhibitor concentrations of 10 μM (Fig. 2D).

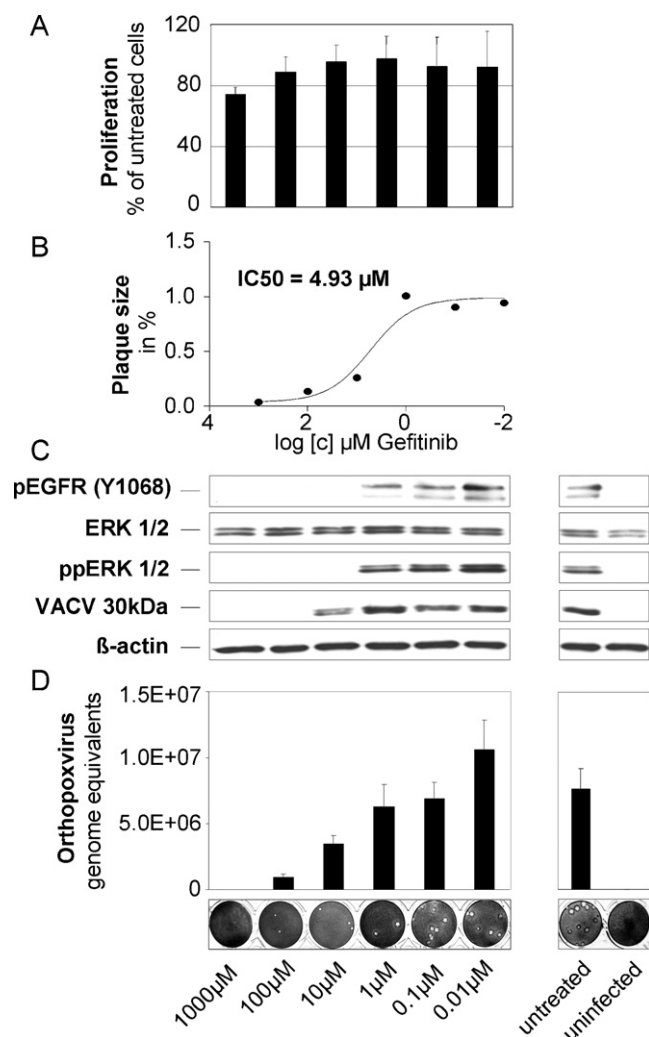
### 3.3. The blocking of EGFR signaling by Gefitinib is dose dependent and non-toxic

Since Gefitinib was shown to be the most effective compound for inhibition of orthopoxvirus spreading correlating with an impaired EGFR signaling the antiviral effect was analyzed for dose dependency in detail. Therefore a plaque reduction test with Gefitinib concentrations ranging from 1000 μM to 0.01 μM was performed. Cells were subsequently analyzed for cytotoxic effects, IC<sub>50</sub> of Gefitinib for plaque size reduction, activated EGFR signaling, viral

protein expression and orthopoxvirus genome equivalents. In order to determine a possible cytotoxicity of Gefitinib a proliferation assay with Alamar Blue was performed measuring the proliferation of infected and treated cells versus the proliferation of infected but untreated cells (Fig. 3A). While for Gefitinib concentrations between 100 μM and 0.01 μM the proliferation rate was almost 100% of the controls, only at concentrations of 1000 μM a slight (24%) decline in proliferation was observed. In contrast proliferation assays for PD153035 and Vandetanib repeatedly revealed a cytotoxic effect at 1000 μM and 100 μM in this assay (data not shown).

IC<sub>50</sub> values of Gefitinib for plaque size reduction were calculated by dividing plaque size by total size of one tissue culture plate well for each compound concentration. A non-linear regression fit curve analysis from the calculated values revealed for plaque size reduction an IC<sub>50</sub> value of 4.93 μM for Gefitinib (Fig. 3B).





**Fig. 3.** Suppression of EGFR signaling by Gefitinib is dose dependent and non-toxic at millimolar concentrations. Results from plaque reduction tests of Hep2 cells infected with VACV (12.5 pfu/well) treated with a serial dilution of Gefitinib (1000–0.01  $\mu\text{M}$ ) and analyzed for (A) proliferation as indicator of cytotoxicity, (B)  $\text{IC}_{50}$  of plaque size inhibition, (C) dose dependent inhibition of EGFR-ERK1/2 signaling and VACV proteins by Western blot analysis (pEGFR detection was obtained from a different experiment with identical settings) and (D) dose dependent inhibition of orthopoxvirus genome replication by real-time PCR. Untreated VACV infected cells and uninfected cells are shown as controls in the right panel.

The analysis of phosphorylated EGFR and of the downstream signaling kinases ERK1/2 in cell lysates of the assay confirmed the dose dependency of the antiviral effect of Gefitinib. Kinase activities were completely suppressed at concentrations above 1  $\mu\text{M}$  as determined by Western blot analysis. VACV proteins were not detectable between 10  $\mu\text{M}$  and 100  $\mu\text{M}$  in Western blot analysis anymore (3C). These observations also were supported by real-time PCR analysis of orthopoxvirus genome copies in cell lysates, revealing a decrease of viral genome copies in correlation with an increase of Gefitinib concentrations (3D).

#### 3.4. Plaque development is accompanied by a distinct and prominent zone of highly EGFR activated but uninfected cells

To analyze the role of activated EGFR in plaque development over time, an immunofluorescent staining for pEGFR and orthopoxvirus proteins in a VACV plaque assay was performed at 24, 48, 72 and 96 h after infection compared to uninfected, untreated cells (Fig. 4A). Therefore the primary antibody against

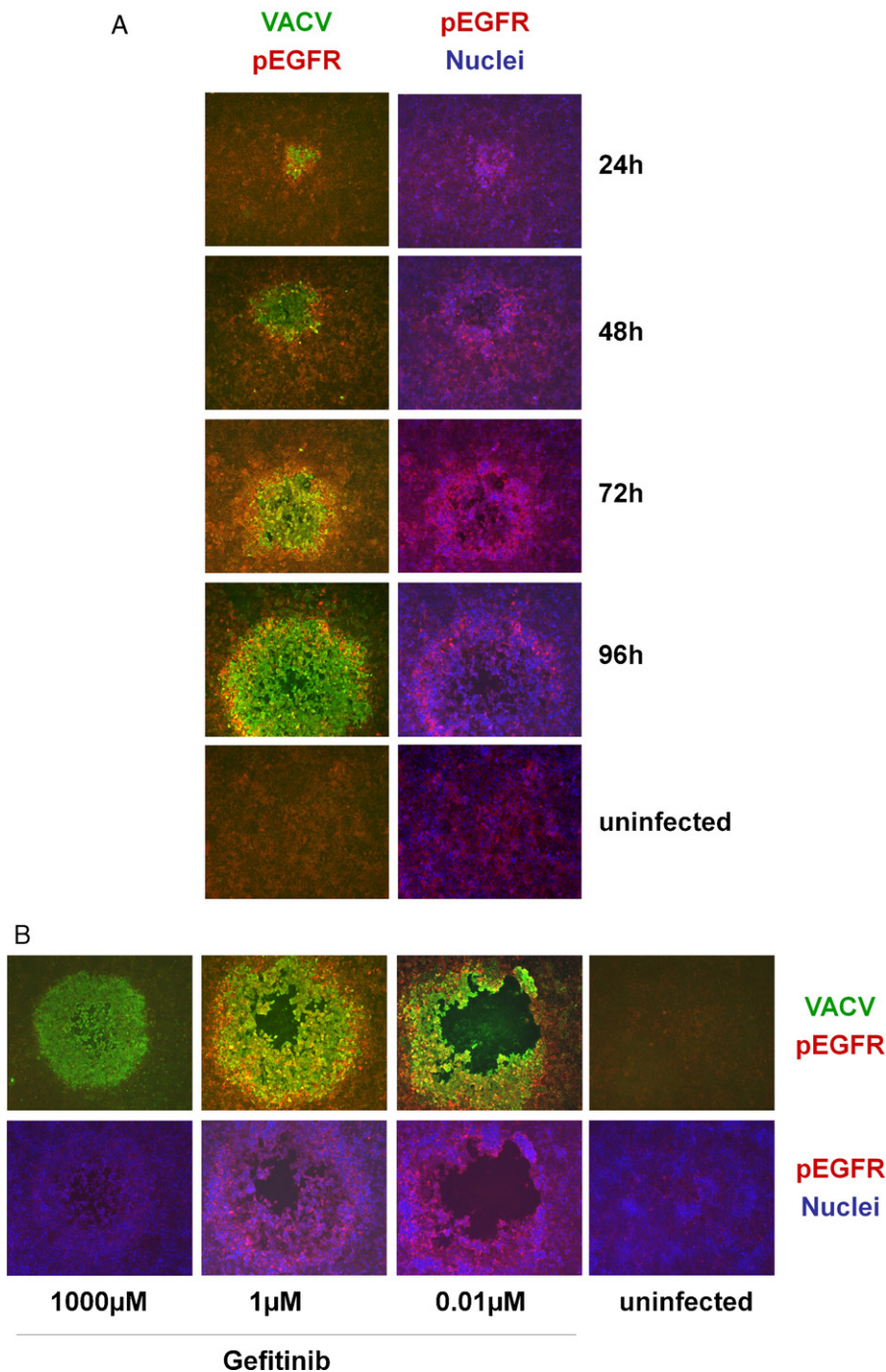
phosphorylated EGFR was detected with cy3 (red) and human immunoglobulins directed against orthopoxvirus proteins with FITC (green) conjugated secondary antibodies. Cellular nuclei were counterstained with DAPI reagent (blue). 24 h after initial VACV infection a germ center of viral spreading could be observed, already showing some twenty infected cells surrounded by disseminated uninfected but pEGFR positive cells. 48–72 h after infection the plaque size increased over time by circular spreading. The cells in the center remained positive for VACV but were negative for pEGFR. These cells were surrounded by a small ring of infected and pEGFR positive cells (merged, yellow), followed by a prominent and distinct zone of uninfected cells but also with activated EGFR. Beyond this zone the EGFR phosphorylation status is similar to uninfected control cell cultures (72 h after infection). 96 h after infection the viral plaques further increased in size and cells in its center were still positive for virus but not for phosphorylated EGFR. At this time point cells in the plaque center were already detached or lost forming a plaque visible to the naked eye. The zone of cells positive solely for pEGFR is rarely visible and the plaque is gated by a small ring of infected and pEGFR positive cells (Fig. 4A).

#### 3.5. The plaque surrounding zone of EGFR activated cells is completely suppressed by effective Gefitinib concentrations

In order to analyze the effect of Gefitinib treatment on the development of VACV induced plaque formation as shown in Fig. 4A, an immunofluorescent staining of a plaque reduction test was performed 96 h after viral infection. VACV infected cells were treated with 1000  $\mu\text{M}$ , 1  $\mu\text{M}$  or 0.01  $\mu\text{M}$  Gefitinib and compared to uninfected cells after 96 h incubation. At concentrations of 1000  $\mu\text{M}$  Gefitinib virus induced plaques were rarely found in cell culture and infected cells only could be identified by fluorescent staining of orthopoxvirus proteins (green) but not by light microscopy. At this Gefitinib concentration EGFR phosphorylation was completely suppressed in the cells and even the uninfected, untreated control cells show a stronger pEGFR signal (control, red). Occasionally found plaques, visible by fluorescence microscopy only, showed a very compact shape with minimal disruption in its center. At the lower Gefitinib concentrations plaques showed an increasing number of infected and pEGFR positive cells (merge, yellow) and a surrounding small zone of solely pEGFR positive cells (red). The lower the Gefitinib concentration the more plaques with a disrupted center were observed (1  $\mu\text{M}$  and 0.01  $\mu\text{M}$ ). Most of the cells localized in the center of the plaque were positive for orthopoxvirus infection but negative for pEGFR (Fig. 4B).

## 4. Discussion

In the present study we analyzed three small molecule inhibitors of EGFR tyrosine kinase activity for their ability to inhibit the spreading of orthopoxviruses. PD153035 and Vandetanib are two compounds that are still either in experimental or in clinical investigation for anti-cancer therapy. The third one, Gefitinib (Iressa<sup>TM</sup>) already successfully passed clinical development and was approved by FDA and EMEA (Cohen et al., 2003; Sequist et al., 2008) as a drug for NSCLC treatment. Previously only one study analyzed EGFR small molecule inhibitors for the purpose of an antiviral chemotherapy against poxviruses. They found the experimental EGFR inhibitor CI-1033 as an effective inhibitor of orthopoxvirus infection (Yang et al., 2005). The same group also described antiviral effects of monoclonal antibodies directed to the Smallpox Growth Factor (SPGF) (Kim et al., 2004). In accordance with that data Postigo et al. showed indirectly the activation of EGFR (anti-P-Tyr antibody 4G10) upon VGF stimulation leading to downstream signaling in the MEK-ERK1/2 pathway (Postigo et al., 2009). It was previously



**Fig. 4.** A circular zone of EGFR activated but uninfected cells facilitate virus induced plaque development, efficiently blocked by Gefitinib. (A) Immunofluorescent staining of a plaque reduction tests with Hep2 cells 24, 48, 72 and 96 h after VACV infection (6.25 pfu/chamber). VACV infected cells (green) were visualized by human IgG directed to orthopoxviruses (VIG) detected with a secondary FITC labelled antibody. Uninfected cells with activated EGFR signaling (red) are detected with a secondary Cy3 labelled antibody. Positive cells for both, VACV and phosphorylated EGFR are shown in yellow (merge). Cellular nuclei are counterstained with DAPI. (B) Immunofluorescent staining of a plaque reduction test as described for (A) and treated with three different Gefitinib concentrations (1000  $\mu$ M, 1  $\mu$ M, and 0.01  $\mu$ M). The test was analyzed 96 h after VACV infection. Uninfected cells are shown as controls. Magnification 100 $\times$ . (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

reported that the MEK-ERK1/2 signaling is required for virus multiplication (Andrade et al., 2004).

Here we show for the first time directly the inhibition of EGFR phosphorylation at tyrosine residue Y1068 leads to reduced orthopoxvirus spreading in epithelial cells by all three inhibitors tested. In consequence blocking of EGFR phosphorylation leads to the loss of ERK1/2 downstream signaling in a dose dependent manner.

As a functional outcome of an abrogated EGFR signaling a reduction in orthopoxvirus induced plaque size was observed previously (Postigo et al., 2009; Yang et al., 2005). In this study we not only observed a significant reduction in plaque sizes but in addition also a statistically significant reduction of plaque numbers when treating VACV infected cells with Gefitinib (IC<sub>50</sub> = 4.93  $\mu$ M).

For a better understanding of EGFR signaling in orthopoxvirus infections we carried out an immunofluorescence staining for

orthopoxvirus proteins and phosphorylated EGFR during the course of viral plaque development in epithelial cells. Thereby for the first time we could visualize a circular zone of cells with highly activated EGFR signaling but negative for orthopoxvirus infection. This prominent zone of activated cells is surrounding orthopoxvirus infected but pEGFR negative cells forming the center of the plaque. The largest expansion of this zone was observed 72 h after infection. Thus we hypothesize that EGFR is activated in a paracrine manner by orthopoxvirus positive cells for subsequent infection. Most likely this paracrine signaling is facilitated by VGF expression in infected cells acting to prime surrounding cells for subsequent infection by anti-apoptotic, pro-proliferative and mitogenic signaling as described previously (Buller et al., 1988b; Opgenorth et al., 1983; Jost et al., 2001a,b; Yarden, 2001). At effective doses Gefitinib efficiently suppressed the involvement of this peripheral EGFR signaling zone surrounding the plaque during development resulting in significant reduction of plaque sizes and plaque numbers. These results are in accordance with recently published data showing that EGFR tyrosine phosphorylation facilitates uptake of VACV by macropinocytosis and actin rearrangement, demonstrating that EGFR is required for the initial entry of the virus into uninfected cells (Mercer et al., 2010). However the authors also provide evidence that EGFR is not the receptor for VACV entry in host cells.

Taken together Gefitinib is the most effective inhibitor among all three small molecules tested in blocking virus spreading in CPXV and VACV infected epithelial cells in a dose dependent and non-toxic manner. Since Gefitinib (Iressa<sup>TM</sup>) is a clinically approved drug for anti-tumor treatment it might be used as an off label medication for the treatment of acute poxvirus infections if primary therapy options, e.g. cidofovir, are inefficient or not indicated. In addition to that targeting a cellular target may reduce the possibility of drug resistance and it remains to be elucidated if combination therapies with antiviral drugs might show an increased efficacy in treating such infections.

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