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Inhibition of poxvirus spreading by the anti-tumor drug Gefitinib (Iressa™)

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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 nulceosid 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™) which is already approved as a drug for anti-tumor medication in the USA and in Europe. Thus Gefitnib 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.

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 (Jahrling 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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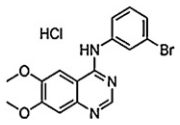
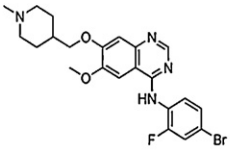
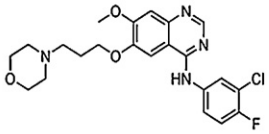
Name	**PD 153035**	**Vandetanib** ZD6474, Zactima	**Gefitinib** Iressa
Mechanism	EGFR tyrosine kinase inhibitor	EGFR / VEGFR tyrosine kinase inhibitor	EGFR tyrosine kinase inhibitor
Molecular Formula			
IC50 Values	≤ 1µM (Bos et al., 1997)	3.5µM - 80µM (Azzariti et al., 2006)	7.3µM - >100µM (Azzariti 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, IC50 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™ 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™ (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_2 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

8×10^4 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_2, 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_2. Cells were overlaid with 200 µl of 1.6% CMC and incubated for further 96 h at 37 °C and 5% CO_2. 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).