Antiviral activity of formyl peptide receptor 2 antagonists against influenza viruses

Noémie Courtin, Aurélien Fotso Fotso, Pierre Fautrad, Floriane Mas, Marie-Christine Alessi, Béatrice Riteau[*]

Aix Marseille Univ, INSERM, INRA, NORT, UMR 1260/1062, Marseille, France

ARTICLE INFO

ABSTRACT

Influenza viruses are one of the most important respiratory pathogens worldwide, causing both epidemic and pandemic infections. The aim of the study was to evaluate the effect of FPR2 antagonists PBP10 and BOC2 on influenza virus replication. We determined that these molecules exhibit antiviral effects against influenza A (H1N1, H3N2, H6N2) and B viruses. FPR2 antagonists used in combination with oseltamivir showed additive antiviral effects. Mechanistically, the antiviral effect of PBP10 and BOC2 is mediated through early inhibition of virus-induced ERK activation. Finally, our preclinical studies showed that FPR2 antagonists protected mice from lethal infections induced by influenza, both in a prophylactic and therapeutic manner. Thus, FPR2 antagonists might be explored for novel treatments against influenza.

© 2017 Elsevier B.V. All rights reserved.

1. Introduction

Influenza is a major public health problem (Kuiken et al., 2012). Annually, influenza epidemics cause 200—500,000 fatal cases worldwide. Viruses of animal origin (mainly avian) can occasionally be transmitted to humans and become pandemic, whose impact can range from mild to severe (40 million deaths for the Spanish Flu). The etiological agents of the disease, the single-stranded RNA influenza viruses, are classified into four types (A, B, C and D). Both influenza A (IAV) and B viruses are responsible for epidemics. IAV are further divided into subtypes based on their surface glycoproteins, hemagglutinin (HA, 18 subtypes) and neuraminidase (NA, 11 subtypes).

Current antiviral drugs used for these pathogens are limited to two approved classes of compounds that target viral proteins, thereby promoting selection pressure. The adamantane compounds block the viral M2 ion channel protein, whereas oseltamivir and zanamivir bind the viral enzyme neuraminidase (NA). Particularly regarding adamantanes, a drastic increase in viral resistance has occurred in recent years (Hayden and de Jong, 2011). The substitution of a single amino acid can make a mutant virus resistant without affecting its virulence (Hayden and de Jong, 2011). This illustrates the urgent need for novel antiviral approaches. In this manuscript, to overcome this challenge of resistance, a cellular molecule was targeted instead of the virus.

The Formyl Peptide Receptors (FPRs) belong to the G protein-coupled receptors (Le et al., 2002), in which three FPRs with similarities in their amino acid sequences, were described in humans (FPR1, 2 and 3). FPR2 also known as FPRL1 (Formyl Peptide receptor-like 1) or ALX (lipoxin A4 receptor) binds different kinds of ligands: formyl peptides, whose major biological source is bacteria, fatty acid lipid mediators [such as lipoxin A4 (LXA4)] and cellular proteins (such as Annexin-1). FPRs might be important receptors in viral pathogenesis. Indeed, FPR1 is activated by the nonstructural protein 5A of hepatitis C viruses inducing activation and migration of human phagocytes (Lin et al., 2011). Regarding FPR2, it is used by immunodeficiency viruses (IV) as a co-receptor for viral replication (Nedellec et al., 2009) both for human IV-1 isolates (Shimizu et al., 2008a) and simian IV (Shimizu et al., 2008b). In addition, the gp41 and gp120 of HIV-1 activate FPR2 on human phagocytes and monocytes, leading to activation and desensitization of cell immune response, respectively (Deng et al., 1999; Su et al., 1999). More recently, we found that FPR2 has a proviral role during IAV infections and increases virus pathogenesis (Tcherniuk et al., 2016). Inhibiting FPR2-signaling with the FPR2 antagonist WRW4 inhibited IAV replication and protected mice from lethal IAV infections. Altogether, these reports illustrate how FPR2 can be used by several viruses to support their own replication.

* Corresponding author. Aix Marseille Univ, INSERM, INRA, NORT, Marseille, France.
  E-mail address: beatrice.riteau@univ-amu.fr (B. Riteau).

The aim of this study was to go further into the identification of molecules targeting FPR2 in order to foster the development of FPR2 antagonists as antiviral molecules against influenza. The present report shows that the FPR2 antagonists PBP10 and BOC2 (Ortiz-Munoz et al., 2014; Skovbakke et al., 2015), are two novel potent antiviral inhibitors of both influenza A and B viruses. Thus, FPR2 is a potential tractable target for treating a broad range of influenza viruses.

2. Materials and methods

2.1. Viruses, cells and reagents

IAV A/PR/8/34 (H1N1), A/HK/68 (H3N2) and influenza B virus B/70 were a gift from GF. Rimmelzwaan (Erasmus Medical Center, Rotterdam, the Netherlands). A/Turkey/Massachussets/65 (H6N2) was a gift from V. Jestin (Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail, Maisons-alfort, France). The human alveolar A549 and the Madin-Darby canine kidney (MDCK) cell lines used in this study were a gift from GF. Rimmelzwaan. Cells were cultured as previously described (Berri et al., 2014). The following reagents were used in the study: monoclonal anti-tubulin antibody (Sigma Aldrich), polyclonal anti-p-ERK antibody (Cell Signaling Technology), anti-viral M2 protein (Santa Cruz), and oseltamivir (Sigma-Aldrich). The ERK inhibitor pathway U0126 was obtained from Sigma-Aldrich.

WRW4 (Trp-Arg-Trp-Trp-Trp-Trp-NH$_2$) is a selective antagonist of FPR2. It inhibits the binding of WKYMVm (FPR2 agonist) to FPR2, resulting in the complete inhibition of ERK signaling as well as intracellular calcium increase (Bae et al., 2004). WRW4 was obtained from Alomone Labs. PBP10 (RhoB-Glu-Arg-Leu-Phe-Glc-Val-Lys-Glc-Arg-Arg) is a 10-aa-long rhodamine-linked and membrane-permeable peptide inhibitor. It adopts a phosphatidy-linositol 4,5-bisphosphate-binding sequence in the cytoskeletal protein gelsolin. Its activity might depend on its ability to pass membrane, disassemble actin filament structures and FPR2-mediated cellular response (Cunningham et al., 2001). It is highly specific to FPR2 and has no inhibitory function on FPR1. PBP10 was obtained from Tocris. BOC2 (Boc-Phe-Leu-Phe-Leu-Phe-OH) is a competitive antagonist of the binding of formyl peptides to FPR. BOC2 blocks both human and murine FPR2 (Verriere et al., 2012; Vital et al., 2016). It also inhibits FPR1 signaling. BOC2 was obtained from CliniSciences.

2.2. Infection experiments and cell viability

A549 cells were pre-incubated for 20 min with or without the indicated concentration of FPR2 antagonist, WRW4, PBP10 or BOC2 before being infected with the indicated influenza virus (MOI 1). In some experiments, assays were performed in the presence of 0.25 μM U0126 or vehicle. After one hour, the virus was removed and medium containing the above- mentioned FPR2 inhibitors was added in the presence or absence of the indicated concentrations of oseltamivir to let virus replication proceed. At the indicated time point post-infection, infectious virus titers were assessed in the supernatant or RNA was extracted. Cell viability in the presence of FPR2 antagonists was assessed by trypan blue staining 72 h post-treatment.

2.3. Real-time quantitative PCR analyses

Total RNA was extracted for each experimental condition from A549 cells using QIAzol reagent (Qiagen) according to the manufacturer's protocol. 5 μg of the resulting RNA was then reverse transcribed using the M-MLV Reverse Transcriptase kit

(Invitrogen). Regarding vRNA (viral RNA) reverse transcription, Uni12 primer was used as previously described (Hoffmann et al., 2001). A specific primer for GAPDH was used for reverse transcription (Baier et al., 1993) as housekeeping gene. Real-Time qPCRs were then performed with the 5X HOT Pol EvaGreen qPCR Mix Plus (Invitrogen). Amplification plots were generated using the Light-Cycler 480 software (Roche), and fold induction was calculated using the threshold cycle method ($2^{-\Delta\Delta Ct}$), GAPDH was used for normalization.

Primer sequences were the following:

2.4. Virus production and titration

Virus production was performed on MDCK cells that were seeded at 15×10^6 cells per 75 cm^2 tissue culture flask and incubated at 37 °C overnight. The next day, cells were infected with IAV at a multiplicity of infection (MOI) of 10-3 in medium containing 1 μg/ml of trypsin. Two days post-infection, the supernatant was harvested, purified by centrifugation and subsequently the virus particles were frozen at −80 °C. For viral titration, MDCK cells were grown in 6 well culture plates and infected with serial dilutions of the supernatant containing the infectious viruses for one hour, at 37 °C. After adsorption, cells were overlaid with medium containing 2% agarose and 1 μg/ml of trypsin and incubated for 3 days, at 37 °C. Viral plaques were then visualized by crystal violet staining.

2.5. Fluorescence microscopy experiments

Fluorescence microscopy was performed as previously described (Berri et al., 2014). Briefly, A549 cells were seeded and cultured on glass coverslips in a multiwell plate. The next day, infection experiments in the presence or absence of PBP10 or BOC2 were performed as described above. Cells were then fixed and permeabilized with 4% paraformaldehyde containing 0,2% Triton-X100. Cells were then extensively washed with phosphate-buffered saline and were incubated with a viral anti-M2 primary antibody for 1 h at 37 °C. Subsequently, an Alexa Fluor (Life Technologies) secondary antibody was used for 1 h at 37 °C. Cells were counterstained with DAPI for 15 min. Images were analyzed using a Zeiss IMAGER.M1.

2.6. ERK activation experiments

Regarding the kinetic of virus-induced ERK phosphorylation, A549 cells were incubated with IAV A/PR/8/34 (MOI 10) at the indicated time point before cell lysis. Regarding the effect of PBP10 or BOC2, A549 cells were first pretreated for 20 min at 37 °C with FPR2 antagonists PBP10 or BOC2. Cells were then incubated with A/PR/8/34 virus (MOI 10) for 5 min and then lysed in ice-cold lysis buffer (1% Triton X-100, 100 mM Tris-HCl [pH 7.4], 1.5 M NaCl, 5 mM EDTA, in the presence of a complete proteinase inhibitor mixture). Proteins from the lysates were then analyzed by western blot, as previously described (Riteau et al., 2003).

2.7. In vivo experiments

Five- to six-week-old female C57BL/6 mice (Charles River) were anesthetized with Ketamine/Xylazine (43/5 mg/kg) and inoculated intranasally with 20 μl of a solution containing A/PR/8/34 virus, as previously described (Berri et al., 2013; Le et al., 2015). Regarding BOC2 treatment (4 mg/kg), mice were treated once the day of virus inoculation (500 PFU). Prophylactic treatment with WRW4 (8 mg/kg) or BOC2 (4 mg/kg) was achieved by treating mice once, one day before virus inoculation (750 PFU). WRW4 and BOC2 were both administered intraperitoneally. Upon virus inoculation, survival