Short communication

Replication of human coronaviruses SARS-CoV, HCoV-NL63 and HCoV-229E is inhibited by the drug FK506

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\begin{abstract}
Recent research has shown that Coronavirus (CoV) replication depends on active immunophilin pathways. Here we demonstrate that the drug FK506 (Tacrolimus) inhibited strongly the growth of human coronaviruses SARS-CoV, HCoV-NL63 and HCoV-229E at low, non-cytotoxic concentrations in cell culture. As shown by plaque titration, qPCR, Luciferase- and green fluorescent protein (GFP) reporter gene expression, replication was diminished by several orders of magnitude. Knockdown of the cellular FK506-binding proteins FKBP1A and FKBP1B in CaCo2 cells prevented replication of HCoV-NL63, suggesting the requirement of these members of the immunophilin family for virus growth.
\end{abstract}

Coronaviruses cause severe diseases of the respiratory and gastrointestinal tract and the central nervous system in animals (Perlman and Netland, 2009). Infection of humans with HCoV-OC43 and HCoV-229E are known since the 1960s to be associated with respiratory tract i.e. common cold diseases. Severe Acute Respiratory Syndrome-Coronavirus (SARS-CoV) is the most aggressive human agent, causing the lung disease SARS (Drosten et al., 2003a). HCoV-NL63 and HCoV-HKU1 were discovered in 2004 and 2005, respectively (van der Hoek et al., 2004; Woo et al., 2005). They cause upper and lower respiratory tract infections including bronchiolitis and pneumonia especially in young children, immunocompromised patients and the elderly (van der Hoek, 2007).

Until now effective treatment of coronavirus infection in humans is unavailable (Stockman et al., 2006), even though inhibitors of coronavirus enzymes (reviewed by (Tong, 2009a,b) and compounds inhibiting in vitro replication have been described (Kono et al., 2008; te Velthuis et al., 2010; Vincent et al., 2005). Coronaviruses represent the group of RNA viruses with the largest RNA genome known to date. Compared to other RNA viruses, the coronavirus polymerase has a rather low error frequency and may possess a RNA proof reading activity (Denison et al., 2011). However, the development of resistant mutants in the presence of drugs targeting coronaviral proteins remains a serious concern. Virus replication depends on a variety of host factors (de Haan and Rottier, 2006; Vogels et al., 2011; Zhang et al., 2010), which in turn represent potential antiviral targets. These might be more preferable targets than viral proteins as development of resistance is much less likely.

In a recent study we performed a genome-wide SARS-CoV yeast-two-hybrid interaction screen with human cDNA libraries identifying cyclophilins and FK506-binding proteins as interaction partners of SARS-CoV non-structural protein 1 (Nsp1). We identified Cyclosporin A (CsA) as a potent replication inhibitor of various human and animal CoVs (Pfefferle et al., 2011). Inhibition of SARS-CoV, HCoV-229E and, in addition, of Mouse Hepatitis Virus was also confirmed by de Wilde et al. (de Wilde et al., 2011). Cyclophilins and FK506-binding proteins are collectively referred to as immunophilins because they bind the immunosuppressive drugs CsA and FK506, respectively. Furthermore, they

\begin{keyword}
SARS-CoV
HCoV-NL63
HCoV-229E
FK506
Tacrolimus
Immunophilins
FKBP1A (FKBP12)
FKBP1B (FKBP12.6)
Inhibition of viral replication
\end{keyword}
share peptidyl-prolyl isomerase (PPlase) domains and chaperone functions facilitating protein folding (Davis et al., 2010). CsA was recently shown to exert inhibitory effects on herpes simplex virus, vaccinia virus (Damaso and Keller, 1994), BK polyoma virus (Acott et al., 2008), human immunodeficiency virus 1 (HIV-1) (Briggs et al., 1999; Wainberg et al., 1988) and HCV (Fischer et al., 2010; Nakagawa et al., 2004; Watashi et al., 2003). Whereas cyclophilins were reported to be required for replication of HIV-1 (Franke and Luban, 1996), vesicular stomatitis virus (Boze et al., 2003) and HCV (Nakagawa et al., 2005), FK506-binding proteins were not found to play a role in the replication of HIV-1 (Briggs et al., 1999) and HCV (Nakagawa et al., 2004; Watashi et al., 2003). Orthopoxviruses are inhibited by FK506 (Reis et al., 2006).

FK506 was one of the first macrolides, discovered in 1984 in the bacterium Streptomyces tsukubaensis, with immunosuppressive activity. Although it is structurally unrelated to the cyclic undecapeptide CsA both molecules display similar properties of binding to the catalytic pocket of the PPIase domains of their cellular binding partners resulting in inhibition of their PPIase activities (Barik, 2006). Furthermore, the cyclophilin-CsA and FKBP-FK506 complexes bind to the cellular phosphatase calcineurin (CnA) which inhibits the dephosphorylation and activity of the nuclear factor of activated T-cells. The inhibition of this essential transcriptional regulator of important immune genes is the cause of immunosuppression. It is important to note that binding of the FKBP/FK506 complex to CnA, which is required for immunosuppression, and inhibition of PPIase activity, which is responsible for antiviral activity, are two independent mechanisms.

When studying the interaction of the SARS-CoV Nsp1 protein with cyclophilins A and B we discovered that replication of various coronaviruses is sensitive to CsA treatment including HCoV-NL63 and HCoV-229E (Pfefferle et al., 2011). Since Nsp1 also interacted with FK506-binding proteins FKBP1A and FKBP1B, we examined in this study whether FK506 inhibits replication of human coronaviruses. In a first experiment VeroFM cells were infected with SARS-CoV at a multiplicity of infection (MOI) of 0.001 in the presence of increasing concentrations of CsA and FK506. Virus RNA concentrations were measured after 24h in supernatant of infected cells by real-time RT-PCR (Drosthen et al., 2003b). Titers were reduced 128,740-fold by 33 μM CsA, and 11,112 fold by 49 μM FK506, respectively. Fig. 1A shows the percentage of SARS-CoV inhibition under the influence of FK506, with an EC50 inhibitory concentration of about 6.9 μM. In Fig. 1B the corresponding log10 titer reductions are represented. To examine whether FK506 exerts an inhibitory activity on other human coronaviruses, CaCo2 cells were infected with HCoV-NL63 at MOI = 0.004 (Herzog et al., 2008) in the presence of increasing inhibitor concentrations of CsA and FK506. Virus RNA concentrations were measured after 24h in supernatant of infected cells by real-time RT-PCR (Drosthen et al., 2003b). Titers were reduced 128,740-fold by 33 μM CsA, and 11,112 fold by 49 μM FK506, respectively. The determined EC50 was 12.2 μM for FK506 under these conditions. GFP fluorescence was inversely correlated to inhibitor concentration, with DAPI staining of cell nuclei indicating confluent of HuH7 cells and equivalent cell densities at all concentration steps. Virus growth was completely abolished between concentration of 8–12 μM CsA and 18–25 μM FK506. The latter correlates well with the inhibitory concentration determined for HCoV-229E-Luc. For both HCoV-229E and HCoV-NL63, EC50 values were higher in plaque and GFP titrations as compared to direct replication assays by either RT-PCR or luciferase measurement. However, deviations between both assay formats were only by factors of 3.2 (HCoV-NL63) and 2.6 (HCoV-229E).

In order to examine whether the cellular FK506-binding proteins FKBP1A and FKBP1B are required for virus replication, CaCo2 knockdown cell lines were established using lentiviral expression of shRNA (Sirion GmbH, Martinsried, Germany). Cells were transduced at MOI 30 with MISSIONTM lentiviral non-target control, cyclophilin B (PPIB), FKBP1A, or FKBP1B shRNA lentiviruses. Transduction was performed at MOI 30 with MISSIONTM lentiviral non-target control, cyclophilin B (PPIB), FKBP1A, or FKBP1B shRNA lentiviruses. Sequences used for gene knockdown are listed in Table 1A. Stable shRNA-expressing cells were generated through 3 weeks of bulk-selection in 10–15 μg/ml puromycin-containing medium (DMEM + 10% FCS + 2 mM l-glutamine + 1 mM Na-pyruvate). The high puromycin concentration needed for selection of CaCo2 cells might result from an enhanced expression of MDR-1 (multi drug resistant protein 1 gene) depleting cells very efficiently from inhibitory drug molecules (Takara et al., 2002). From the bulk-selected knockdown and control cells mRNA expression was quantified by real-time RT-PCR. For reverse transcription 1 μg of total RNA was used. Amplification products were detected by SYBR I, and amplicon integrity was verified by melting point analysis. Cyclophilin A (PPIA) served as a reference gene against PPIB to determine the specificity of the knockdown. mRNA expression levels of PPB1, FKBP1A, and FKBP1B were then determined by real-time RT-PCR with primers listed in Table 1B. Fig. 3A shows PPB1, FKBP1A and FKBP1B mRNA expression in bulk-selected CaCo2 cells. In comparison to non-target controls a 98% and 96% knockdown was determined for PPB1 and FKBP1A/1B, respectively.

Plaque titration showed that HCoV-NL63 grew to similar titers in CaCo2 wild type (not shown), CaCo2 shRNA control and CaCo2-ΔPPB1 knockdown cells indicating that expression of PPB1 was not necessary for viral growth. Conversely, knockdown of FKBP1ΔA/B
Fig. 1. Effect of FK506 on SARS-CoV in VeroFM and on HCoV-NL63 replication in CaCo2 cells by qPCR (A–D) and plaque titration (E and F). Data shown are mean values of representative experiments performed in at least triplicates. Left Y-axes represent the percentage of reduction of virus replication in linear scale (A, C and E) and in log scale (B, D and F) at the indicated inhibitor concentrations, respectively. Percentage of cell viability with the mock-treated cells set to 100% are shown on the right Y-axes. FK506 concentrations used for each virus are given on the X-axis.

did not result in any plaque formation, identifying FKBP1A/B products as key proteins required for viral replication. Similarly, real-time PCR amplification of RNA genomes in the supernatant of the HCoV-NL63-infected cell lines showed significantly reduced titers in the CaCo2-ΔFKBP1A knockdown cells as compared to CaCo2sh and CaCo2-ΔPPIB knockdown cells (not shown), confirming the results of the plaque titrations.

In summary, FK506 inhibits the replication of SARS-CoV, HCoV-NL63 and HCoV-229E at non-toxic, low-micromolar concentrations with a reduction of virus titers by several orders of magnitude to undetectable levels. In a comparative study of the effect of CsA and FK506 on HIV-1, the former inhibited virus replication completely [IC50: 1–2 μg/ml] whereas latter had an inhibitory effect on chronically but not on newly infected cells [up to 10 μg/ml FK506 (Briggs et al., 1999)]. Also in the case of HCV, FK506 had no effect on the replication of replicon RNA up to concentrations of 3 μg/ml (Watashi et al., 2003). Considering the higher drug concentrations of CsA and FK506 needed for inhibition of coronavirus replication it can be speculated that also HIV-1 and HCV might be inhibited by higher FK506 concentrations. It also remains to be clarified why the low nanomolar affinities of FKBP1A/B to FK506 do not reflect the EC50 inhibitory concentrations of coronaviruses which reside
in the low micromolar range. This might be explained by a stronger affinity of coronavirus proteins to the cellular proteins as compared to FK506.

Lack of growth of HCoV-NL63 in stable CaCo2-ΔFKBP1A and -ΔFKBP1B, but not -ΔAPP1B knockdown mutants indicates the requirement of FKBP1A and FKBP1B for HCoV-NL63 replication. As the two proteins share about 83% homology on the amino acid level (not shown) they do not substitute for each other upon individual knockdown. A common structural feature of FKBP proteins is the PPIase domain, also called FK506-binding domain. Its peptidylprolyl cis/trans isomerase activity influences cellular pathways by binding to cellular proteins (Kang et al., 2008). These activities are common to many FKBP family members containing a PPIase domain and separate chaperone functions can be discriminated from them (Barik, 2006).

The two less pathogenic coronaviruses HCoV-NL63 and HCoV-229E and the highly pathogenic SARS-CoV are sensitive to CsA and FK506, indicating the involvement of cyclophilins and FK506-binding proteins in viral replication. However, it is not clear what functions of these cellular proteins are required for virus replication. For SARS-CoV we have shown that Nsp1 binds to cyclophilins and to FKBP1A and FKBP1B by Y2H and Lumier assay (Pfefferle et al., 2011). Also, Nsp1 proteins of HCoV-NL63 and -229E interact with FKBP1A and FKBP1B in Y2H (not shown). The mechanism and
Table 1

| Particle set | Target shRNA sequence (5′ → 3′) | shRNA sequence (5′ → 3′) |
|--------------|----------------------------------|--------------------------|
| Non-target control | TRC1.5 Vector (pLKO.1-puro) | CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTTTTG |
| PPIB: SHVRS-NM | TRCN000049251 | CCGGCTTCTTCTACAGACAGTCAACTCGAGCTGACTGTCGTGATGAAGAICTTTTG |
| FKBP1A: SHVRS-NM | TRCN000000595 | CCGGCGGAAATCTGATATATCTCCACTCGAGTGGAGATATAGTCAGTTTGGCTTTTT |
| FKBP1B: SHVRS-NM | TRCN00000151663 | CCGGGAAGTTTGATTCATCCAGAGACTCGAGTCTCTGGATGAATCAAACTTCTTTTG |

(A) shRNA sequences used for lentiviral-based gene knockdown

(B) Sequences of primers used for quantification of gene knockdown

| Primer | Sequence (5′ → 3′) |
|--------|-------------------|
| hPPIB_F | CTCTCCGAACGCAACATGAAG |
| hPPIB_R | ACCTTGACGGTGACTTTGGG |
| hFKBP1A_F | CATCACCACGGGATCTCGTTAG |
| hFKBP1A_R | TTCTTCCCAGCCTCGGATCA |
| hFKBPB_F | GCAGGAAGGAACTCAAGGTG |
| hFKBP1B_R | AGCAACTTGGGCAGAGAGAA |

function of these interactions have to be analysed in more detail. Furthermore it has to be clarified whether other viral proteins are involved in the relation of coronaviruses and immunophilins.

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