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Fluoroquinolones inhibit human polyomavirus BK (BKV) replication in primary human kidney cells

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ABSTRACT

Reactivation of human polyomavirus BK (BKV) may cause polyomavirus-associated nephropathy or polyomavirus-associated hemorrhagic cystitis in renal- or bone marrow-transplant patients, respectively. Lack of treatment options has led to exploration of fluoroquinolones that inhibit topoisomerase II and IV in prokaryotes and possibly large T-antigen (LT-ag) helicase activity in polyomavirus. We characterized the effects of ofloxacin and levofloxacin on BKV replication in the natural host cells – primary human renal proximal tubular epithelial cells (RPTECs). Ofloxacin and levofloxacin inhibited BKV load in a dose-dependent manner yielding a ~90% inhibition at 150 μg/ml. Ofloxacin at 150 μg/ml inhibited LT-ag mRNA and protein expression from 24 h post infection (hpi). BKV genome replication was 77% reduced at 48 hpi and a similar reduction was found in VP1 and agnoprotein expression. At 72 hpi, the reduction in genome replication and protein expression was less pronounced. A dose-dependent cytostatic effect was noted. In infected cells, 150 μg/ml ofloxacin led to a 26% and 6% inhibition of cellular DNA replication and total metabolic activity, respectively while 150 μg/ml levofloxacin affected this slightly more, particularly in uninfected cells. Cell counting and xCELLigence results revealed that cell numbers were not reduced. In conclusion, ofloxacin and levofloxacin inhibit but do not eradicate BKV replication in RPTECs. At a concentration of ofloxacin giving ~90% inhibition in BKV load, no significant cytotoxicity was observed. This concentration can be achieved in urine and possibly in the kidneys. Our results support a mechanism involving inhibition of LT-ag expression or functions but also suggest inhibition of cellular enzymes.

1. Introduction

The majority of humans have a persistent polyomavirus BK (BKV) infection in their renourinary tract (Hirsch and Steiger, 2003). Intermittently, the virus reactivates, replicates in epithelial cells and is shed in urine. In immunocompetent individuals, reactivation is self-contained and gives no symptoms or permanent damage (Egli et al., 2009). However, in individuals with profound immune dysfunction such as kidney transplant patients and allogenic hematopoietic stem cell transplant recipients, the replication of BKV may be extremely high and this together with other factors may cause severe damage to the kidney graft or to the urinary bladder leading to polyomavirus-associated nephropathy (PyVAN) and polyomavirus-associated hemorrhagic cystitis (PyVHC), respectively (Dropulic and Jones, 2008; Hirsch, 2010). For PyVAN, reduction of immunosuppression can be tried but is not always sufficient and cannot be used for all patients. Some patients are treated with the nucleotide analog cidofovir or the pyrimidine synthesis inhibitor leflunomide but a recently published systemic review on treatment of PyVAN concluded that there was no graft survival benefit of adding these drugs (Johnston et al., 2010). Our own *in vitro* studies found that their anti-BKV activities were due to cytostatic effects (Bernhoff et al., 2008, 2010). Unfortunately, there are no randomized control studies and the benefit of such treatment is controversial (Rinaldo and Hirsch, 2007).

Fluoroquinolones, a family of synthetic broad spectrum antimicrobial agents, targets bacterial enzymes topoisomerase IV and topoisomerase II (Andriole, 1994). Already in 1988, the fluoroquinolones norfloxacin, coumermycin, nalidixic- and oxolinic-acid were shown to inhibit BKV DNA replication and progeny production in African green monkey kidney (Vero) cells (Portolani et al., 1988; Ferrazzi et al., 1988). Later, studies performed in human embryonic lung fibroblast cell lines HEL and WI-38 reported a strong to modest anti-BKV effect of ciprofloxacin (Leung et al., 2005; Randhawa, 2005). In a small study with 10 renal transplant patients, gatifoxacin was found to reduce BKV viremia and viruria (Trofe et al., 2006) but ciprofloxacin was not found to protect renal

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transplant patients from BKV reactivation the first year post transplantation (Koukoulaki et al., 2008). However, ciprofloxacin was reported to reduce the incidence of BKV viruria in hematopoietic stem cell transplant patients (Leung et al., 2005) and a retrospective study of renal transplant patients given levofloxacin or ciprofloxacin as prophylaxis for Pneumocystis showed significantly lower rates of BKV viremia up to 1 year post transplantation (Gabardi et al., 2010). One clinical trial is now investigating the effect of 30 days of oral levofloxacin in renal transplant patients with BKV viremia (ClinicalTrials.gov NCT01034176), while another is planning to investigate the effect of 3 months of levofloxacin prophylaxis in renal transplant patients (ClinicalTrials.gov NCT01353339).

Fluoroquinolones have been suggested to interfere with the helicase activity of BKV large T antigen (LT-ag) protein as reported for the similar Simian virus (SV) 40 LT-ag (Ali et al., 2007). LT-ag is an early regulatory multifunctional protein that has prominent functions in the lifecycle of BKV. In addition to interfering with the function of cellular regulators including p53 and Rb, it binds to the origin of replication of BKV DNA and orchestrates its bidirectional replication in the presence of host cell proteins such as DNA polymerase α-primase and topoisomerase I (Cuesta et al., 2010). The helicase activity of LT-ag seems to be crucial for separation of the double-stranded DNA genome during the replication. If fluoroquinolones can inhibit LT-ag without overt cytotoxicity to the host cells, these drugs may be of great benefit for many affected patients.

The aim of our study was to investigate the effect of fluoroquinolones ofloxacin and levofloxacin on BKV replication in renal tubular epithelial cells, the primary target cells in PyVAN.

2. Materials and methods

2.1. Cell and virus

Primary human renal proximal tubule epithelial cells (RPTECs) (Lonza, www.lonzabioscience.com) were propagated as described by the manufacturer. No latent BKV could be detected by PCR of intracellular DNA. All experiments were performed with RPTECs at passage 4 and BKV-Dunlop supernatants or gradient purified virus both obtained from Vero cells.

2.2. Infection and drug treatment

Ofloxacin (Sigma, www.sigmaaldrich.com) (Fig. 1A) and levofloxacin (Sigma) (Fig. 1D) were dissolved to 2 mg/ml in RPTEC growth medium and further diluted to working concentrations. RPTECs at about 50% confluence were infected with BKV-Dunlop for 2 h before removing infectious units and adding growth medium with or without ofloxacin or levofloxacin, unless indicated otherwise.

2.3. Cell viability and cell proliferation assay

Cellular DNA replication was quantified by colorimetric measurement of BrdU incorporation into DNA (for 20 h) using Cell proliferation ELISA, BrdU kit (Roche, www.roche-applied-science.com). Total cellular metabolic activity was monitored by colorimetric measurement of reduction of resazurin (Res) dye (for 3 h) by mitochondrial, microsomal and cytosolic enzymes using TOX-8 (Sigma). Cell adhesion, proliferation and size were monitored in real-time using the xCELLigence RTCA SP instrument (Roche) and expressed together as Cell index (CI) as previously described (Rinaldo et al., 2010). In short, 29 h after seeding, half of the media was replaced with fresh media with or without purified BKV-Dunlop in the presence of ofloxacin (final concentration: 0, 150 or 400 μg/ml). The CI was measured every 15 min for the first 6 h after seeding and thereafter every 30 min.

2.4. RNA extraction and cDNA synthesis

Cells were lysed and total RNA was extracted using mirVana™ miRNA isolation kit (Ambion, www.ambion.com) followed by DNase treatment using TURBO DNA-free™ Kit (Ambion). The quality and concentration of RNA was measured by Nanodrop. cDNA was synthesized from 150 ng RNA using High Capacity Reverse Transcription kit (Applied Biosystems, www.appliedbiosystems.com) and expression level of BKV mRNA was quantified in triplicate by reverse transcription quantitative PCR (RT-qPCR) as described previously (Bernhoff et al., 2008). The housekeeping gene human Hypoxanthine PhosphoRibosylTranserase (huHPRT) was insignificantly affected by ofloxacin at 24, 48 and 72 hpi and was used for normalization.

2.5. Preparation of DNA

For extracellular BKV loads, cell culture supernatants were harvested at 72 hpi and frozen at −70 °C until analysis by qPCR. For intracellular BKV loads, cells were washed, trypsinized, pelleted and resuspended in G2 buffer from MagAttract DNA Mini M48 kit (Qiagen, www.qiagen.com) and frozen at −70 °C until automatic extraction (GenoM-48, Qiagen).

2.6. Quantitative PCR for BKV DNA and cellular gene detection

To quantitate extracellular or intracellular BKV DNA loads, each sample was analyzed in triplicate by qPCR with primers and probe targeting the BKV LT-ag gene (Hirsch et al., 2001). To express intracellular BKV DNA as Geq/cell, each sample was analyzed simultaneously by qPCR for the gene aspartoacylase (ACY) (Randhawa et al., 2002; Bernhoff et al., 2008).

2.7. Western blotting

Cells were lysed in cell disruption buffer (mirVana™ miRNA isolation kit, Ambion), collected and stored at −70 °C until separation with SDS–polyacrylamide gel electrophoresis (SDS–PAGE) followed by blotting onto PVDF (polyvinylidene fluoride) membrane. BKV and cellular proteins were detected and quantified as previously described (Rinaldo et al., 2010). In addition, polyclonal rabbit anti-N-terminal LT-ag (1:1000) (Hey et al., 1994) was used.

2.8. Immunofluorescence staining, microscopy and digital image processing

Immunofluorescence staining was performed as previously described (Rinaldo et al., 2010). Images were collected using a Nikon TE2000 microscope and processed with NIS-Elements BR 3.2 (Nikon Corporation). For cell counting, 5 pictures per well were randomly taken using 10× objective. The Draq5 and LT-ag stained nucleus were counted automatically by ImageJ (http://rsbweb.nih.gov/ij/), and then corrected manually. For confocal microscopy, cells were grown in chamber slides, fixed in 4% paraformaldehyde and then permeabilized by methanol. Immunofluorescence staining was performed as before (Rinaldo et al., 2010). The cells were examined by Zeiss Laser Scanning Microscope 510 Meta (Carl Zeiss, Inc.) with a C-Apochromat 40×/1.4 W objective.

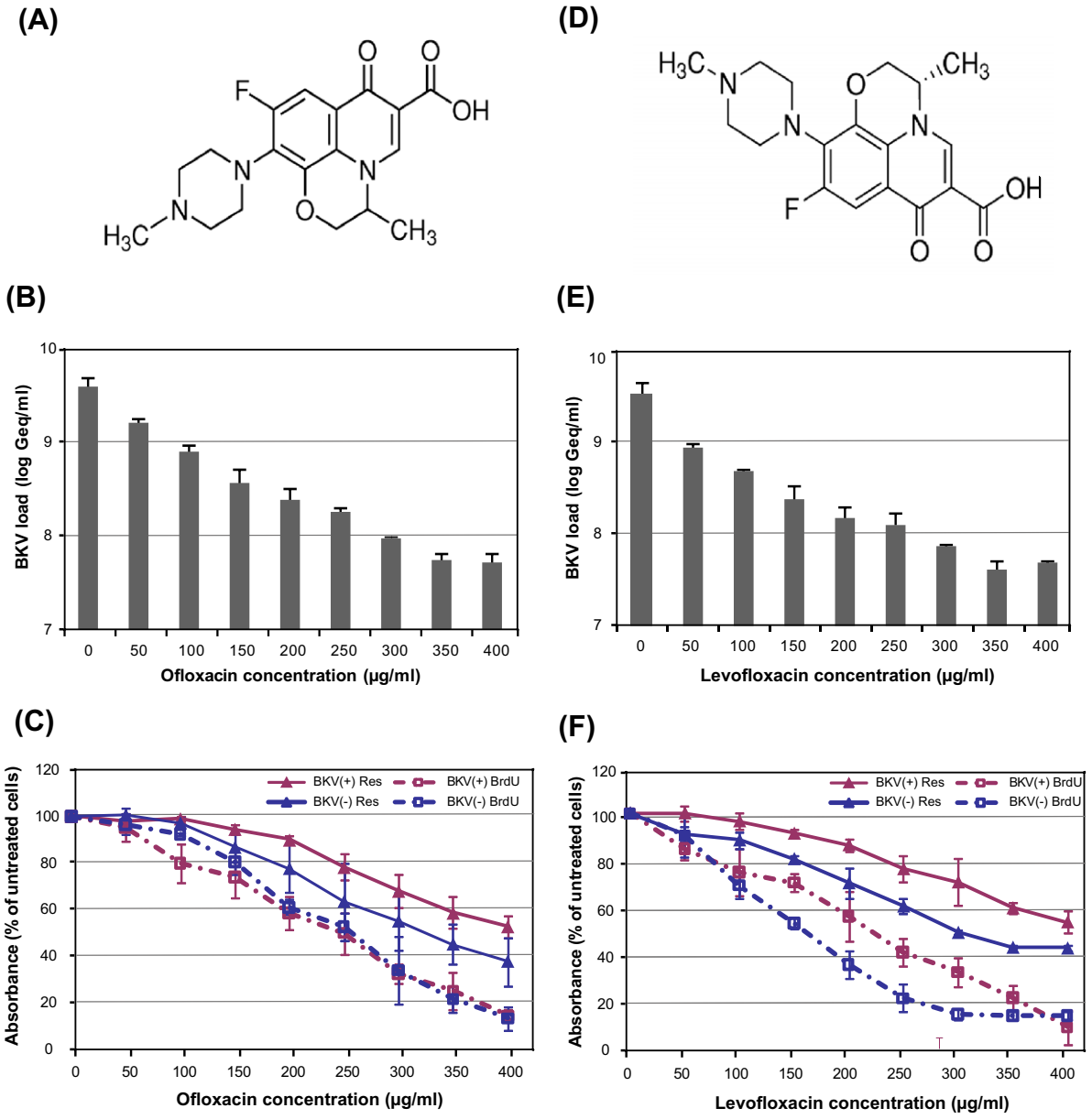

Fig. 1. Effect of increasing concentrations of ofloxacin and levofloxacin on BKV load and cell viability. The chemical structures of ofloxacin and levofloxacin are shown in (A) and (D), respectively. Supernatants were harvested at 72 hpi from BKV-infected RPTECs treated with indicated concentrations of ofloxacin (B) or levofloxacin (E) and BKV loads were measured by qPCR, respectively. Mean values ± SD of two experiments (each experiment was performed in two wells) are presented as Geq (Genome equivalent)/ml. Cellular DNA replication (BrdU) and total metabolic activity (Resazurin) of ofloxacin (C) and levofloxacin (F) treated uninfected (BKV−) and BKV-infected (BKV+) RPTECs were measured at 72 hpi. Mean values ± SD of two experiments (each experiment was performed in three wells) are presented as percent of absorbance of untreated cells.

2.9. Infectious progeny virus release

The supernatants harvested from untreated and ofloxacin-treated BKV-infected RPTECs at 72 hpi were used to infect new RPTECs for 2 h before removing the supernatants, washing and addition of fresh growth medium. At 72 hpi, the cells were washed, methanol-fixed and immunostained as described above.

2.10. Selectivity index of ofloxacin and levofloxacin

Extracellular BKV load and cellular DNA replication (uninfected RPTECs) measured at 72 hpi were used to calculate percent of inhibition. The results were analyzed by XLfit program (Fit Model: Dose response one site 210) to determine effective concentration of ofloxacin and levofloxacin for 50% inhibition of BKV load (EC_{50}) and for 50% inhibition of cell cytotoxicity (CC_{50}) monitored as cellular DNA replication. The selectivity index (SI_{50}) was calculated by dividing CC_{50} by EC_{50}. In addition, EC_{90} for 90% inhibition of BKV load was calculated.

3. Results

3.1. Effect of ofloxacin and levofloxacin on BKV load and cell viability

First, the effect of ofloxacin on BKV load was investigated. RPTECs were infected with BKV and increasing concentrations of ofloxacin were added at 2 hpi. Supernatants were collected and cells were methanol-fixed at 72 hpi. Thereafter, BKV loads in supernatants were measured by qPCR. Ofloxacin decreased extracellular BKV load in a concentration dependent manner and at

150 μg/ml (415 μM) the BKV load was inhibited by ~90% (Fig. 1B). Next, the effect of ofloxacin on cellular DNA replication and total metabolic activity was investigated. As above, ofloxacin was added and cellular DNA replication and total metabolic activity were measured at 72 hpi. We found that ofloxacin reduced both events in a concentration-dependent manner (Fig. 1C) and compared to untreated cells, ofloxacin at 150 μg/ml reduced cellular DNA replication and total metabolic activity by 26% and 6% in infected cells and by 20% and 13% in uninfected cells, respectively. Immunofluorescence staining of fixed cells demonstrated a clear concentration-dependent reduction of cells expressing the early protein LT-ag and the late non-structural agnoprotein of unknown function (Fig. 2A). When the total cell number and infected (LT-ag stained) cells were counted, 58% of untreated cells were infected but only 24% of cells treated with ofloxacin at 150 μg/ml (Fig. 2B). Of note, ofloxacin at concentrations 50–150 μg/ml increased the total cell number (up to 20%) while higher concentrations gradually reduced it (Fig. 2B).

The anti-BKV effect of levofloxacin was similarly investigated. Levofloxacin decreased extracellular BKV load (Fig. 1E), cellular DNA replication and total metabolic activity (Fig. 1F) in a concentration-dependent manner. Levofloxacin at 150 μg/ml (415 μM) reduced BKV load by ~90%; and cellular DNA replication and total metabolic activity by 30% and 8.6% in infected cells; and 47% and 20% in uninfected cells, respectively. Immunofluorescence staining revealed that levofloxacin had a similar concentration-dependent inhibition of the number of BKV-infected RPTECs as ofloxacin (data not shown). In conclusion, both ofloxacin and levofloxacin inhibited BKV replication in a concentration-dependent manner. Both drugs also affected cellular DNA replication and total metabolic activity, but to a lesser extent than BKV replication. Of note, levofloxacin seemed to be more cytostatic to uninfected than infected cells. According to a clinically validated mathematical model, BKV replication must be inhibited by >90% in order to clear viremia and viruria within 3 and 10 weeks, respectively (Funk et al., 2008). As such we selected the concentration of the least cytostatic drug that gave a 90% reduction of extracellular BKV load. Accordingly, ofloxacin at 150 μg/ml was selected for our further experiments.

3.2. Effect of ofloxacin on uninfected and BKV-infected RPTECs monitored in real-time

Ofloxacin reduced cellular DNA replication and total metabolic activity at 72 hpi. To investigate this in real-time, we examined uninfected and infected RPTECs with or without ofloxacin from the time of seeding and the next 104 h. Ofloxacin at 150 μg/ml increased the cell index (CI) while at 400 μg/ml decreased it in both uninfected and BKV-infected cells (Fig. 3). Taken together with the microscopic evaluation, we concluded that ofloxacin at 150 μg/ml slightly increased the cell number but possibly also the cell size and/or adhesion of both uninfected and BKV-infected cells, while a concentration of 400 μg/ml decreased these parameters.

3.3. Effect of ofloxacin on BKV early gene expression

To investigate the effect of ofloxacin on BKV early gene expression, we measured LT-ag mRNA levels at 24, 48 and 72 hpi by RT-qPCR and normalized this to the expression of a house keeping gene. The result was presented as fold expression relative to the untreated sample at 24 hpi. In addition, we also performed Western blot on cell extracts harvested at the same time points. In untreated cells LT-ag mRNA expression was increased by more than 90-fold from 24 to 72 hpi. Ofloxacin reduced this expression from 1.0 to 0.65-fold (35% reduction) at 24 hpi, whereas at 48 and 72 hpi the reduction was 16.6-fold (60%) and 50.6-fold (50%), respectively, compared to the untreated cells (Fig. 4A). At 24 hpi, Western blot for early proteins LT-ag and st-ag repeatedly demonstrated their expression only in untreated but not in ofloxacin treated cells. Compared to untreated cells, the expression of LT-ag was reduced by 35% and 20% and of st-ag by 60% and 40% at 48 and 72 hpi, respectively (Fig. 4C). Although LT-ag was not detectable by Western blot at 24 hpi in ofloxacin-treated cells, confocal microscopy of immunofluorescence stained cells revealed a low proportion of LT-ag expressing cells (data not shown). We conclude that ofloxacin inhibited both early transcription and expression and that the decrease was strongest at 48 hpi and more moderate at 24 and 72 hpi.

3.4. Effect of ofloxacin on BKV genome replication

To investigate whether BKV genome replication in RPTECs, starting around 36 hpi, was affected by ofloxacin, we measured intracellular BKV DNA loads at 24, 48 and 72 hpi by qPCR and normalized this to the cell number using the amount of a simultaneously measured cellular gene. The results are presented as Geq/cell. Compared to untreated cells, ofloxacin was found to reduce the BKV load by 77% at 48 hpi and 58% at 72 hpi (Fig. 4B). We conclude that ofloxacin significantly reduced BKV genome replication.

3.5. Effect of ofloxacin on BKV late gene expression

To determine the effect of ofloxacin on BKV late gene expression, we performed Western blot on cell extracts at 24, 48 and 72 hpi. As expected, the late VP1 and agnoprotein were not detected before 48 hpi and were then reduced by 85% and 80% and at 72 hpi by 40% and 30%, respectively (Fig. 4D). A similar reduction was observed in late mRNA expression (data not shown). Confocal microscopy of immunofluorescence stained cells at 72 hpi confirmed our previous finding of a reduced number of BKV-infected cells but also revealed that the staining pattern was somewhat changed. While the nuclei of most untreated infected cells had strong VP1 inclusions, this was changed by ofloxacin (Fig. 4E) similar to previously described for leflunomide and CMX001 treated cells (Bernhoff et al., 2010; Rinaldo et al., 2010). We conclude that ofloxacin reduced late protein expression at 48 hpi and to a lesser extent at 72 hpi.

3.6. Effect of ofloxacin on infectious viral progeny release

In order to study if the decrease in extracellular BKV DNA corresponded to the release of infectious viral progeny, supernatants harvested from BKV-infected untreated and ofloxacin treated RPTECs (72 hpi) were used to infect cells and immunofluorescence staining for BKV proteins performed at 72 hpi. A high percentage of cells inoculated with supernatant from untreated cells were infected but only a few infected cells were observed when supernatants from ofloxacin treated cells were tested (Fig. 4F). By counting the LT-ag expressing cells, a 98% reduction was found. We conclude that ofloxacin 150 μg/ml reduced the release of infectious BKV progeny by more than 90%.

3.7. Effect of later or multiple additions of ofloxacin to BKV-infected RPTECs

We have observed that intracellular BKV DNA and BKV gene expression is more affected at 48 than 72 hpi. We hypothesized that the drug was catabolized and that the effect would be stronger by daily dosing. We were also interested to see how the timing of treatment affected BKV replication. In order to investigate this, we added ofloxacin at 150 μg/ml to BKV-infected RPTECs once (at 2 or 24 hpi); twice (at 2 and 24 or 2 and 48 hpi) and three times (at 2,

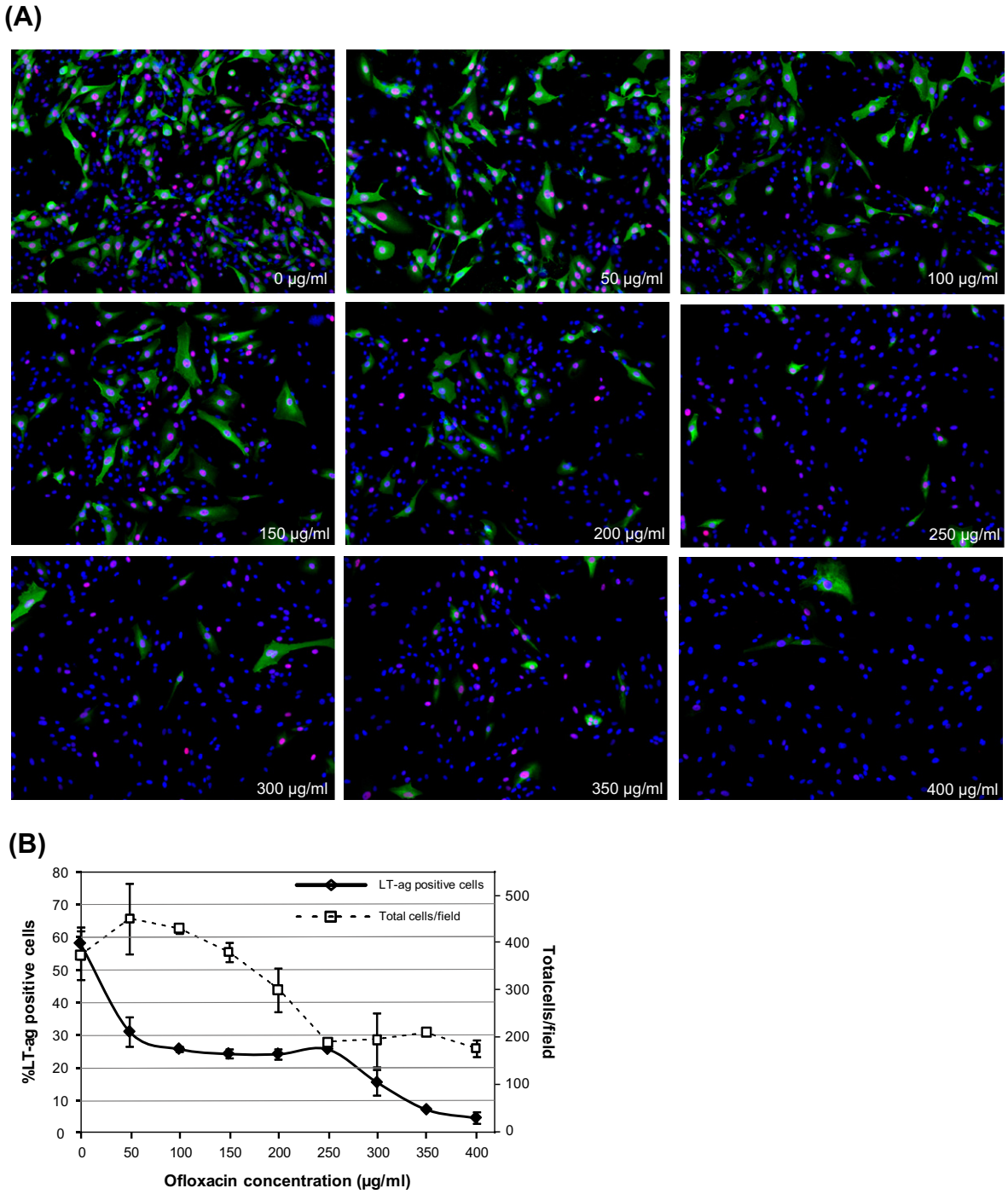

Fig. 2. Effect of increasing concentrations of ofloxacin on expression of BKV early and late proteins. (A) Indirect immunofluorescence of BKV-infected RPTECs treated with the indicated concentrations of ofloxacin. The cells were fixed at 72 hpi and stained with polyclonal rabbit anti-agnoprotein serum for visualization of BKV late agnoprotein (green) and with the SV40 LT-ag monoclonal antibody Pab416 for visualization of BKV early protein LT-ag (red). Cell nuclei (blue) were stained with Draq5. The pictures were taken with a fluorescence microscope (10× objective). (B) The total cell number and percent of LT-ag stained cells in the pictures were counted by ImageJ. Mean values ± SD of two experiments are presented.

24 and 48 hpi). As before, extracellular BKV loads in supernatants, cellular DNA replication and total metabolic activity were measured at 72 hpi. Compared to untreated cells, addition of ofloxacin at 2 or 24 hpi decreased the BKV load by 1.4 and 0.8 log, respectively. The reduction in cellular DNA replication and total metabolic activity of the same treatment was proportional to the decrease in BKV load (Fig. 5). The addition of ofloxacin twice at 2 and 24 hpi or at 2 and 48 hpi reduced the BKV load by 1.8 or 1.6 log, respectively, whereas the decrease in BKV load was 1.8 log when ofloxacin was added three times. However, similar increased inhibition was also seen in cellular DNA replication and total metabolic activity by these treatments compared to administration of ofloxacin once at 2 hpi. We conclude that early addition of ofloxacin had stronger effect on extracellular BKV load than later addition and that several additions decreased the extracellular BKV load more than addition once but that this also induced more cytostatic effects in host cells. The latter result suggests that no significant catabolism is taking place during the experiment.

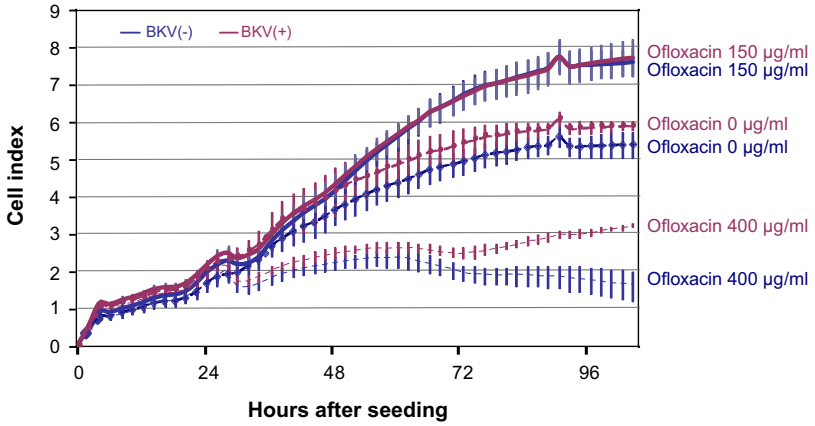

Fig. 3. Effect of ofloxacin on adhesion, proliferation and size of uninfected and BKV-infected RPTECs. RPTECs were seeded in E-plate (6000 cells/well), and 29 h after seeding, half of the medium in the wells were replaced by the growth medium with or without purified BKV-Dunlop and with ofloxacin to reach the indicated drug concentrations. Cell index (CI), a combined measure of cell adhesion, proliferation and size, was monitored from seeding until 104 h (75 hpi) by xCELLigence RTCA SP instrument. The cell indexes (normalized to cell free background) at 2 h interval are shown as mean values ± SD of 6 wells treated with ofloxacin at 0 or 150 µg/ml and of 2 wells with ofloxacin at 400 µg/ml.

3.8. The selectivity index of ofloxacin and levofloxacin

In order to calculate SI_{50}, the EC_{50} and CC_{50} was needed and we therefore modeled the concentration-dependent effect on extracellular BKV loads as well as BrdU incorporation in uninfected RPTECs. The EC_{90} was also calculated. The results were fitted to an exponential decay function. For ofloxacin the EC_{50} was found to be 38 µg/ml and the EC_{90} 152 µg/ml, while the CC_{50} was found to be 249 µg/ml and the CC_{90} was found to be >400 µg/ml (Supplementary Fig. 1). These results suggest a SI_{50} of 6.48 and a SI_{90} of >2.62. For levofloxacin the EC_{50} was found to be 21 µg/ml and the EC_{90} 121 µg/ml, while the CC_{50} was found to be 150 µg/ml and the CC_{90} was found to be >400 µg/ml (Supplementary Fig. 2). These results suggest a SI_{50} of 7.13 and a SI_{90} of >3.29.

4. Discussion

There are at present no antiviral drugs licensed to treat BKV infections. Due to some promising *in vitro* studies on BKV replication, fluoroquinolones have been tried as prophylaxis or treatment of BKV replication in a small number of transplant patients. Our data demonstrate that ofloxacin and levofloxacin both inhibit BKV replication in primary human RPTECs without significant effects on the cell viability. However, the concentrations of drugs and the duration of treatment used did not eradicate BKV. We found the level of early LT-ag transcripts and proteins being reduced by ofloxacin at 24 hpi and as a result, at least partly, all later steps i.e. BKV DNA replication, late gene transcription and expression and finally the amount of infectious progeny released was significantly reduced.

While ofloxacin is formulated as a racemic mixture of S- and R-isomers, levofloxacin is an S-isomer of ofloxacin. A similar pharmacokinetic and toxicity profile has been reported for these drugs even though levofloxacin seems to be twice as active as ofloxacin against prokaryotes (reviewed by Kang et al., 1994). In our study, ofloxacin and levofloxacin inhibited extracellular BKV loads and cell viability in a concentration-dependent manner. The EC_{90} of BKV load was calculated to be 152 µg/ml for ofloxacin and 121 µg/ml for levofloxacin, while their EC_{50} was 38 µg/ml and 21 µg/ml, respectively. So even though their EC_{90} was similar, at lower concentrations, levofloxacin seemed to be almost twice as efficient at reducing BKV loads, consistent with the activity in prokaryotes. Since levofloxacin gave a CC_{50} at a concentration of approximately 50% of ofloxacin, the S-isomer seems to be responsible for the cytostatic effect in RPTECs. The slightly stronger cytostatic effect of levofloxacin on uninfected cells, made us focus on ofloxacin. Despite a different potency, our results suggest that these drugs affect BKV replication in a similar way. Moreover, we have found similar effects using a solution of ciprofloxacin intended for intravenous use (data not shown).

In a productive BKV infection, the first step after uncoating of the BKV genome is the transcription of early pre-mRNA by RNA polymerase II followed by splicing to 3 functional early mRNAs (LT-ag, st-ag and truncated LT-ag). As expected, at 24 hpi (i.e. before the DNA replication has started), we found a very low level of LT-ag mRNA in untreated cells, and this level was reduced by 35% in ofloxacin treated cells. In agreement, a reduction in protein expression was found by Western blot and immunostaining. Early in SV40 infection, low levels of LT-ag protein stimulate LT-ag transcription (Cole, 1996). The same is probably happening during BKV infection. If ofloxacin interacts with LT-ag and thereby inhibits the autostimulation, a slower genome replication, reduced late protein expression and hence a reduced BKV load would be expected. This would probably be similar to replication of BKV variants with a weak early promoter (Gosert et al., 2008; Olsen et al., 2009). In prokaryotes fluoroquinolones make an irreversible ternary complex between the drug, the topoisomerase and DNA (reviewed by Anupama et al., 2010). The smaller reductions in BKV proteins and DNA replication observed at 72 than 48 hpi could indicate a loss of drug activity. However, since multiple drug additions increased the cytostatic effects, the drug seemed to be stable. Multiple additions also decreased the virus load. The fact that ofloxacin when first added at 24 hpi (i.e. after LT-ag was expressed), gave a 84% reduction in BKV load supports that a direct interaction between ofloxacin and LT-ag is involved. Alternatively, ofloxacin has a direct effect on BKV genome replication. In addition to LT-ag, the BKV genome replication is dependent on at least 10 different cellular proteins (Hassell and Brinton, 1996). It is difficult to say if there is any additional direct effect of ofloxacin on later steps since a reduced BKV genome replication automatically reduces late transcription and hence later steps (Cole, 1996). The changed nuclear architecture in ofloxacin-treated infected cells may be explained by the inability of low BKV DNA levels to increase local VP1 concentration (Bernhoff et al., 2010).

Even though our results support the concept that the anti-BKV effect involves an interaction with LT-ag, it may not be the only mechanism. By monitoring ofloxacin and levofloxacin treated

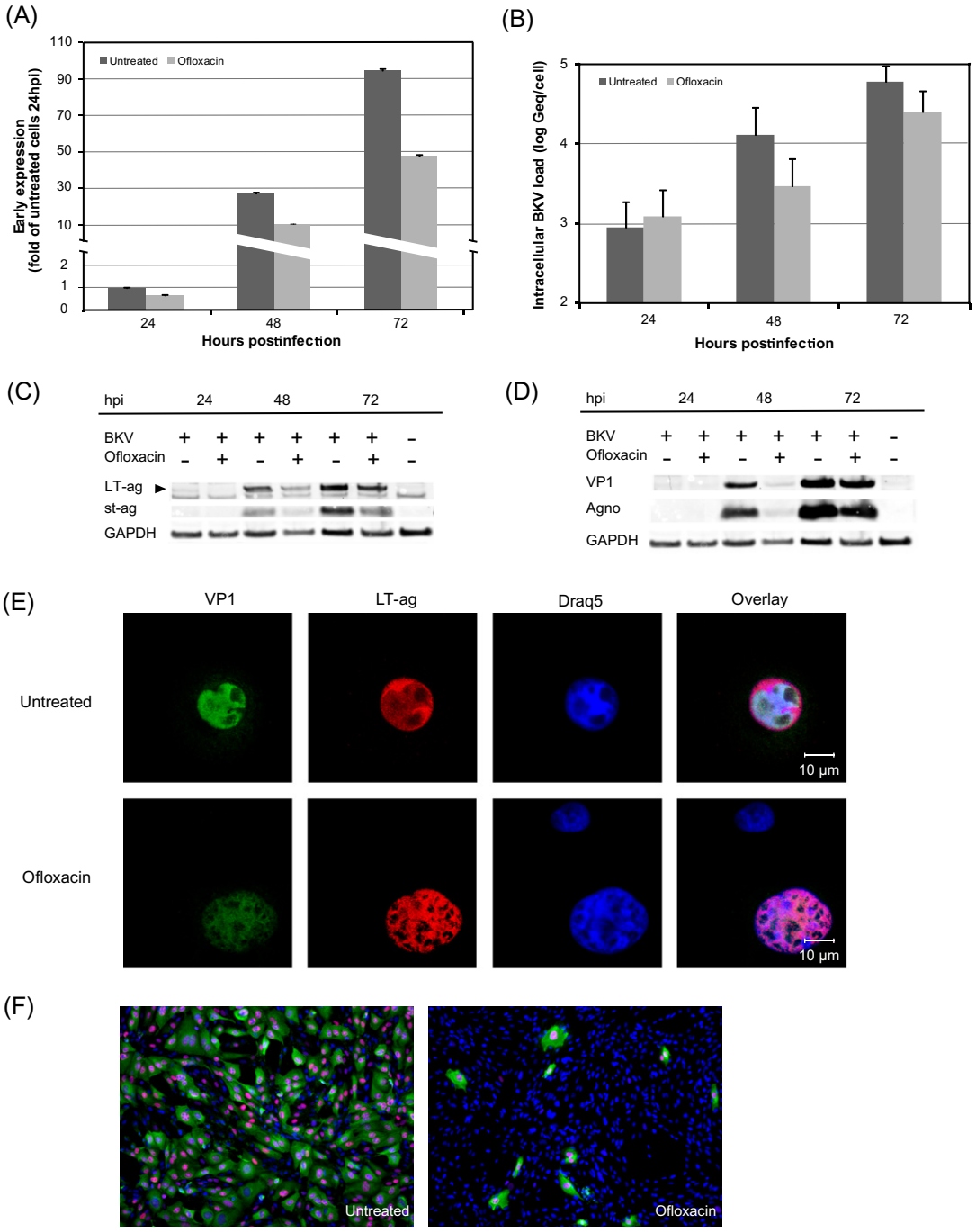

Fig. 4. Effect of ofloxacin at 150 µg/ml on different phases of BKV replication cycle. (A) Effect of ofloxacin on BKV early mRNA expression. RNA was isolated from untreated and ofloxacin-treated BKV-infected RPTECs at indicated time points and LT-ag mRNA levels were measured by RT-qPCR and normalized to huHPRT transcripts. Mean values ± SD of two experiments (each sample was prepared from two wells) are presented as fold expression where the level of untreated cells at 24 hpi is arbitrarily set as 1. To illustrate the difference in LT-ag m-RNA expression in untreated and ofloxacin treated cells at 24 hpi, the *Y*-axis is interrupted and has two different scales. (B) Effect of ofloxacin on BKV genome replication. Untreated and ofloxacin-treated BKV-infected RPTECs were harvested at the indicated time points and DNA was extracted. Intracellular BKV DNA was measured and expressed as Geq/cell. Mean values ± SD of four experiments (each of two experiments was performed in two wells and each of the other two experiments was performed in one well) are presented. (C) Effect of ofloxacin on BKV early protein expression. Cell extracts were prepared from untreated and ofloxacin-treated BKV-infected RPTECs at the indicated time points and Western blot was performed with a polyclonal rabbit anti-N-terminal LT-ag serum and a monoclonal antibody directed against the house keeping protein glyceraldehydes-3-phosphate dehydrogenase (GAPDH). The anti-N-terminal LT-ag serum recognizes LT-ag and st-ag and also a cellular protein of unknown origin. (D) Effect of ofloxacin on BKV late protein expression. Cell extracts were made from untreated and ofloxacin-treated BKV-infected RPTECs at the indicated time points and Western blot was performed with polyclonal rabbit anti-VP1 and anti-agnoprotein serum and monoclonal anti-GAPDH antibodies. (E) Effect of ofloxacin on BKV protein expression in situ. At 72 hpi, cells were fixed and indirect immunofluorescence staining was performed using as primary antibodies polyclonal rabbit anti-VP1 serum (green) combined with the SV40 LT-ag monoclonal antibody Pab416 (red). Cell nuclei (blue) were stained with Draq5. The pictures were taken with a confocal microscope (40× objective). (F) Effect of ofloxacin on infectious viral progeny release. The supernatants from untreated and ofloxacin treated BKV-infected RPTECs were harvested at 72 hpi and seeded onto new RPTECs. At 72 hpi, cells were fixed and indirect immunofluorescence staining was performed using as primary antibodies polyclonal rabbit anti-agnoprotein serum (green) combined with the SV40 LT-ag monoclonal Pab416 (red). Cell nuclei (blue) were stained with Draq5. The pictures were taken with a fluorescence microscope (10× objective).

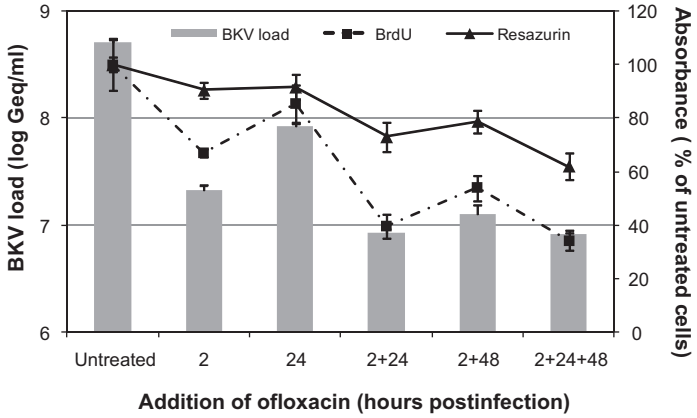

Fig. 5. Effect of multiple additions of ofloxacin to BKV-infected RPTECs. Ofloxacin at 150 μg/ml was added once (2 or 24 hpi) or up to several times as indicated to BKV-infected RPTECs. The supernatants were harvested at 72 hpi and BKV loads were measured by qPCR. Mean values ± SD from two wells are presented as Geq/ml. Similarly, cellular DNA replication (BrdU) and total metabolic activity (Res) were measured at 72 hpi. Mean values ± SD of three wells are shown as percent of absorbance of untreated cells.

RPTECs by BrdU incorporation and resazurin reduction, inhibition was observed in both uninfected and BKV-infected cells. In infected cells BrdU is incorporated into both cellular and viral DNA but the absorbance measured comes mainly from cellular DNA as we have calculated the BKV genomes to constitute only about 5% of the total cellular DNA in a cell containing 6×10^4 BKV genomes. Since cytotoxicity normally is defined as cell lethality of more than 50% (Anupama et al., 2010), ofloxacin at 150 μg/ml is not cytotoxic but cytostatic, at least during the timeframe investigated. In fact, both total cell counting and real-time viability monitoring showed an stimulatory effect of ofloxacin up to 150 μg/ml. Notably, an increased thymidine incorporation has been reported in fluoroquinolone treated Vero cells (Portolani et al., 1988). The inhibition of cellular DNA replication observed in fluoroquinolone treated cells may result from inhibition of the closely related mammalian topoisomerase II (Anupama et al., 2010). We speculate that inhibition of topoisomerase II also reduce BKV genome replication since this enzyme is required to unwind the positive supercoils accumulating ahead of the replication fork and to decatenate the progeny DNA molecules after replication is completed (reviewed by Hassell and Brinton, 1996). Actually, Portolani and colleagures (1988) observed an accumulation of covalently closed supercoiled BKV DNA in fluoroquinolone treated cells and suggested a mechanism involving DNA topoisomerase (Portolani et al., 1988) and a topoisomerase I inhibitor (camptothecin) has been reported to inhibit the DNA replication of the closely related polyomavirus JC (Kerr et al., 1993). Conversely, the concentrations necessary to elicit the inhibition of mammalian topoisomerase II are believed to be rarely achieved *in vivo* (Anupama et al., 2010).

How does the EC_{90} (152 μg/ml) of ofloxacin, compare to concentrations obtained *in vivo*? The mean serum concentration of ofloxacin after twice daily oral dosing of 400 mg is only 6.5 μg/ml (Israel et al., 1993). However, urinary concentration after one single dose has been reported to be 427 μg/ml at 0–6 h and then gradually decrease (Naber et al., 2001). Thus, we can conclude that urinary concentrations are up to 4 times higher than the EC_{90} while serum concentrations are several times lower. To our knowledge, it is not clear from which side ofloxacin is taken up by RPTECs. A study in human intestinal epithelial (Caco-2) cells found transport of ciprofloxacin to be mainly from the basolateral to the apical surface (Cavet et al., 1997a). However, in human airway epithelial (Calu-3) cells, ciprofloxacin was found to be transcellularly transported by passive diffusion at the same magnitude from both sides (Cavet et al., 1997b). If RPTECs behave like the Calu-3 cells, the urinary concentration could be of the same importance as the serum concentration. Besides, the concentration of fluoroquinolones in kidney tissue has been reported to be 2- to 10-fold higher than serum concentrations (Wolfson and Hooper, 1989), indicating either a uptake from urine or a active transport system on the basolateral side.

Both ofloxacin and levofloxacin have been used by millions of people since their introduction in the early 1990s. Since adverse effects usually are mild and rarely result in discontinued treatment (reviewed by Liu, 2010), these drugs may be used for treatment or prophylaxis of BKV disease as soon as their anti-BKV effect is well documented. When calculating the SI_{50} for ofloxacin and levofloxacin, we found this to be only 6.48 and 7.13, respectively. However, we used the sensitive BrdU incorporation assay monitoring cellular DNA replication to calculate this. As discussed previously (Gosert et al., 2011) using a less sensitive assay like resazurin, the SI_{50} for ofloxacin and levofloxacin would have become 8.15 and 14.92, respectively. Using an assay monitoring cell death, the SI_{50} would have become much higher. Of note, a SI_{50} of 1.1 for ofloxacin and 1.7 for levofloxacin was found when BKV-infected WI-38 cells were treated at 2 hpi and intracellular BKV and cellular DNA loads were measured 7 days after the infection (Randhawa, 2005). However, these cells are clearly less relevant than RPTECs.

In conclusion, our results demonstrate that fluoroquinolones can inhibit BKV replication in its natural host cells without causing significant cell toxicity. Our data support that the mechanism for inhibition involves BKV LT-ag, but additional inhibition of host cell proteins like topoisomerase II may be involved.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2011.07.012.

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