Polyomavirus BK (BKV) causes polyomavirus-associated nephropathy (PyVAN) and hemorrhagic cystitis (PyVHC) in renal and bone marrow transplant patients, respectively. Antiviral drugs with targeted activity against BKV are lacking. Since the antimalarial drug artemesunate was recently demonstrated to have antiviral activity, the possible effects of artemesunate on BKV replication in human primary renal proximal tubular epithelial cells (RPTECs), the host cells in PyVAN, were explored. At 2 h postinfection (hpi), RPTECs were treated with artemesunate at concentrations ranging from 0.3 to 80 μM. After one viral replication cycle (approximately 72 hpi), the loads of extracellular BKV DNA, reflecting viral progeny production, were reduced in a concentration-dependent manner. Artesunate at 10 μM reduced the extracellular BKV load by 65%; early large T antigen mRNA and protein expression by 30% and 75%, respectively; DNA replication by 73%; and late VP1 mRNA and protein expression by 47% and 64%, respectively. Importantly, the proliferation of RPTECs was also inhibited in a concentration-dependent manner. At 72 hpi, artemesunate at 10 μM reduced cellular DNA replication by 68% and total metabolic activity by 47%. Cell impedance and lactate dehydrogenase measurements indicated a cytostatic but not a cytotoxic mechanism. Flow cytometry and 5-ethynyl-2'-deoxyuridine incorporation revealed a decreased number of cells in S phase and suggested cell cycle arrest in G0 or G2 phase. Both the antiproliferative and antiviral effects of artemesunate at 10 μM were reversible. Thus, artemesunate inhibits BKV replication in RPTECs in a concentration-dependent manner by inhibiting BKV gene expression and genome replication. The antiviral mechanism appears to be closely connected to cytostatic effects on the host cell, underscoring the dependence of BKV on host cell proliferative functions.
Moreover, artesunate has been reported to be active against cancer cells and parasites (reviewed in reference 27).

The reported broad antiviral activity, coupled with high bioavailability (29) and limited adverse effects (16), made it an interesting candidate for antiviral therapy of BKV infections. Thus, the aim of our study was to perform a detailed investigation of the effects of artesunate on BKV replication in human primary renal proximal tubular epithelial cells (RPTECs), the host cells for BKV during PyVAN.

Here we show that artesunate inhibits BKV replication in RPTECs in a concentration-dependent manner by a mechanism closely connected to its cytostatic effects. Our results also underscore the close relationship between BKV replication and the host cells.

**MATERIALS AND METHODS**

**Cells and virus.** Human RPTECs (ScienCell) were propagated in renal epithelial growth medium (REGM; Lonza) containing 0.5% fetal bovine serum. No latent BKV was detected by quantitative PCR (qPCR) of intracellular DNA. All experiments were performed with RPTECs at passage 4 and BKV Dunlop supernatants or CsCl gradient-purified virus, both obtained from Vero cells. BKV Dunlop has been shown to have a high replication capacity in RPTECs (30) and has been used for several antiviral studies in these cells (12, 13, 31, 32).

**Infection and drug treatment.** Artesunate (Saokim, Hanoi, Vietnam) was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 473 mM and immediately stored in aliquots at −70°C. Prior to use, the stock was diluted in the growth medium to working concentrations. RPTECs at about 50% confluence were incubated with BKV Dunlop for 2 h before surplus infectious units were removed. The RPTECs were washed once with phosphate-buffered saline (PBS), and the medium with or without artesunate was added, unless indicated otherwise. A DMSO control at a concentration matching the DMSO concentration in the 10 µM artesunate dilution was included in all experiments.

**Extraction of RNA and intracellular DNA.** A NucleoSpin TriPrep (total DNA, RNA, and protein isolation) kit (Macherey-Nagel GmbH) was used to isolate RNA and intracellular DNA from the cell lysates prepared from untreated and artesunate-treated cells at 24 and 48 h postinfection (hpi). In brief, the cells were washed once with PBS before a mixture of 350 µl RP1 buffer (provided in the kit) and 3.5 µl β-mercaptoethanol was added to lyse the cells. The cell lysates were collected and stored at −70°C until the isolation of RNA and DNA following the manufacturer’s instructions. The RNA and DNA concentrations were measured by use of a NanoDrop apparatus.

**cDNA synthesis and quantification of BKV mRNA levels.** cDNA was synthesized from 150 ng RNA using a high-capacity reverse transcription (RT) kit (Applied Biosystems), and the level of BKV mRNA was quantified in duplicate by RT-qPCR, as described previously (12). Of note, truncated large T antigen but no small T antigen (st-ag) transcripts were also intercepted by the LT-ag RT-qPCR, but for simplicity, we refer to these as LT-ag transcripts. The housekeeping gene human hypoxanthine phosphoribosyltransferase (huHPRT) was found to be insignificantly affected by artesunate treatment for 24 and 48 h and was therefore used for normalization by the 2^-ΔΔCT threshold cycle (Ct) method (33). The expression at 24 hpi was also normalized to the level of expression of beta-actin (ACTB), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and TATA box binding protein (TBP).

**Quantification of extracellular and intracellular BKV loads.** Cell culture supernatants were harvested and stored at −70°C until the extracellular BKV DNA load was quantified by qPCR using primers and a probe targeting the BKV LT-ag gene (34). Shortly before qPCR, supernatants were diluted in distilled H2O (1:100) and boiled for 5 min. The intracellular BKV DNA load was determined by performing the same qPCR on DNA extracted from cells. To express the amount of intracellular BKV DNA as the number of genome equivalents (GEq) per cell, each sample was simultaneously analyzed by qPCR for the gene aspartoacylase (ACY) (12, 35). All experiments had duplicate samples, and each sample was measured in triplicate.

**Western blotting.** To prepare cell lysates for Western blotting, cells in 48-well plates were solubilized in 50 µl cell dismutation buffer (mirVana microRNA isolation kit; Ambion) at 24 hpi or 100 µl buffer at 48 hpi and stored at −70°C until use. For each sample, 24 µl of cell lysate was separated by SDS-polyacrylamide gel electrophoresis (PAGE) and proteins were blotted onto a polyvinylidene difluoro (PVDF) membrane. Viral and cellular proteins were detected and quantified by the use of a Li-Cor Odyssey imager as previously described (31). The following primary antibodies were used: polyclonal rabbit antisera directed against BKV VP1 (36), BKV agnoprotein (37, 38), and BKV N-terminal LT-ag (37). Anti-serum directed against BKV N-terminal LT-ag detected both LT-ag and st-ag. In addition, a monoclonal mouse antibody directed against the housekeeping protein GAPDH (6C5; Abcam) was used. The protein standard marker was the Odyssey two-color marker (Li-Cor).

**Immunofluorescence staining, microscopy, and digital image processing.** Immunofluorescence staining was performed as previously described (31) using as primary antibodies a polyclonal rabbit antisera directed against BKV agnoprotein (37, 38) and a mouse monoclonal simian virus 40 (anti-SV40) LT-ag antibody (PAb416; Abcam). DNA was labeled with Draq5 dye (Biotatus). Images were captured using a Nikon TE2000 microscope (×10 objective) and processed with NIS-Elements BR (version 3.2) software (Nikon Corporation).

**Infectious progeny virus release.** The supernatants harvested from untreated and artesunate-treated BKV-infected RPTECs at 72 hpi were used to infect new RPTECs for 2 h before removing the inoculum, washing the cells, and adding fresh growth medium. At 72 hpi, the cells were washed, methanol fixed, and immunostained as described above.

**Cell viability and cell proliferation assay.** The viability of mock- and BKV-infected RPTECs with or without artesunate treatment was monitored in real time using an xCELLigence RTCA DP instrument as previously described (31). This system measures the adhesion, proliferation, and size of the cells and expresses these together as an arbitrary unit, the cell index (CI). In short, at 25 h after seeding, half of the medium was replaced with fresh medium with or without purified BKV Dunlop and with or without artesunate. The CI was measured every 15 min for the first 6 h after seeding and thereafter every 30 min.

In addition, cellular DNA replication in mock- and BKV-infected RPTECs with or without artesunate treatment was quantified by colorimetric measurement of bromodeoxyuridine (BrdU) incorporation into DNA (for 20 h) using a cell proliferation enzyme-linked immunosorbent assay (Roche). Total cellular metabolic activity was monitored by colorimetric measurement of the reduction of resazurin (Res) dye (for 3 h) by mitochondrial, microsomal, and cytosolic enzymes using a TOX-8 kit (Sigma).

The potential cytotoxic effects of artesunate on BKV-infected RPTECs were quantified by use of a Cytotoxicity Detection KitPLUS (Roche) following the manufacturer’s instructions. In short, the lactate dehydrogenase (LDH) released from dead cells was measured in supernatants. Similarly, the total LDH (in supernatants and cells) of parallel wells was measured after prior addition of a potent lysis solution provided in the kit. Percent cytotoxicity was calculated by dividing the amount of LDH in the supernatant by the total amount of LDH for the corresponding concentration of artesunate. Each experiment had triplicate samples.

**Analysis of cell cycle by flow cytometry.** Untreated and artesunate-treated RPTECs cultured in 75-cm² flasks were trypsinized, washed twice with PBS, and fixed in 75% ice-cold ethanol at 4°C for at least 24 h. Next, the cells were centrifuged at 300 × g for 5 min, and the ethanol was completely removed without disturbing the cell pellet. The cells were resuspended in PBS with 1% bovine serum albumin (BSA) and incubated at room temperature for 5 min. Thereafter, the cells were centrifuged again at 300 × g for 5 min, washed once with PBS with 1% BSA, resuspended in...
Artesunate inhibits BKV replication in RPTECs. BKV Dunlop requires approximately 48 to 72 h to complete one replication cycle in RPTECs (12). In order to determine the effect of artesunate on BKV replication, cells were infected for 2 h, artesunate was added at 0.3 to 80 µM, and the BKV DNA load in the supernatants was measured by qPCR at 72 hpi. Artesunate decreased the extracellular BKV DNA load in a concentration-dependent manner, with a reduction of up to 1.2 log (i.e., a 93% reduction) (Fig. 1A). Artesunate at 10 µM gave a 65% reduction, and the DMSO control (which gave 13% inhibition) indicated that part of this reduction was due to the DMSO.

Immunofluorescence staining of cells fixed at 72 hpi showed a concentration-dependent reduction in cells expressing the BKV early protein LT-ag (red) and the late agnoprotein (green) (Fig. 1B). Of note, the staining also revealed a gradual reduction in cell numbers (blue) when increasing concentrations of artesunate were used. However, the morphology of the cells seemed to be unchanged with concentrations up to 10 µM (data not shown).

To investigate whether the inhibition of the BKV DNA load in the supernatants also corresponded to a decline in the amount of infectious BKV progeny released, the collected supernatants were used to infect new RPTECs. Again, cells were fixed at 72 hpi and immunofluorescence staining was performed with antibodies directed against LT-ag and agnoprotein. A significant concentration-dependent reduction in the number of cells expressing LT-ag (red) and agnoprotein (green) was seen when supernatants from cells treated with artesunate at 1.25 µM or higher concentrations were used as inocula (Fig. 1C). These results confirmed that artesunate decreased the release of infectious progeny in a concentration-dependent manner. We conclude that artesunate inhibits BKV replication in RPTECs. Since artesunate at 10 µM gave a 65% BKV load reduction without changing the cellular morphology, this concentration was chosen for use in the next experiments, unless otherwise stated.

Artesunate reduces BKV early and late gene expression. To investigate the effect of artesunate on BKV early and late gene expression, we measured LT-ag and VP1 mRNA in untreated and artesunate-treated RPTECs by RT-qPCR at 24 and 48 hpi. The

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**FIG 1 Effect of increasing artesunate concentrations on BKV replication in RPTECs.** (A) Extracellular BKV DNA load. At 72 hpi, supernatants were harvested from BKV-infected RPTECs treated with artesunate at the indicated concentrations from 2 hpi, and BKV DNA loads were measured by qPCR. Mean values of the number of GEq/ml ± SDs of four experiments (each experiment was performed in two wells) are presented. *, *P < 0.05, determined by the t test. (B) Expression of BKV proteins. At 72 hpi, BKV-infected RPTECs treated with the indicated concentrations of artesunate from 2 hpi were fixed and stained with polyclonal rabbit anti-agnoprotein serum for visualization of the BKV late agnoprotein (green) and with the SV40 LT-ag monoclonal antibody PAb416 for visualization of the BKV early protein LT-ag (red). Cell nuclei (blue) were stained with Draq5. The pictures were taken with a fluorescence microscope (×10 objective). (C) Infectious progeny release. The supernatants from untreated and artesunate-treated BKV-infected RPTECs harvested at 72 hpi were also seeded onto new RPTECs. At 72 hpi, these cells were fixed and indirect immunofluorescence staining was performed, as described for panel B. The pictures were taken with a fluorescence microscope (×10 objective).
DMSO Mock
DMSO Mock +

huHPRT transcripts, and presented as described for panel A. (C) BKV early mRNA levels were measured by RT-qPCR, normalized to the levels of the huHPRT transcripts. Mean fold expression values and LT-ag mRNA levels were measured by RT-qPCR and normalized to the untreated and artesunate-treated BKV-infected RPTECs at 24 and 48 hpi, respectively (Fig. 2A). At 24 hpi, the normalization was also performed with other housekeeping genes (ACTB, GAPDH, and TBP), and this gave similar results (data not shown). At 24 hpi, the mRNA expression of VP1 was, as expected, very low, and no significant differences were found between untreated and artesunate-treated samples. At 48 hpi, artesunate reduced the level of VP1 mRNA expression from 509- to 269-fold, i.e., a reduction of 47% (Fig. 2B). The DMSO control did not affect LT-ag mRNA expression but reduced VP1 mRNA expression by 13%.

Next, we investigated the effect of artesunate on BKV protein expression by Western blot analysis. The same volume of cell lysate from all samples was investigated, and the results were normalized to the expression of the housekeeping protein GAPDH. Compared to untreated samples, 75% and 25% reductions of LT-ag expression were found at 24 and 48 hpi, respectively (Fig. 2C), and a similar reduction was found for sT-ag expression (Fig. 2C). At 24 hpi, very low levels of BKV late proteins were observed, and presumably, VP1 mainly represents input virus. At 48 hpi, both VP1 and agnoprotein were strongly expressed in untreated cells, while artesunate treatment reduced their expression by 64 and 49%, respectively. Of note, artesunate also reduced the expression of GAPDH by 40 and 70% at 24 and 48 hpi, respectively, probably reflecting the reduced cell numbers observed by Draq5 staining after artesunate treatment (Fig. 1B). The DMSO control did not affect LT-ag protein expression but increased VP1 expression by 13% and reduced agnoprotein expression by 3%. We conclude that artesunate has a significant inhibitory effect on the expression of early and late BKV proteins. For the early proteins, the inhibition is more pronounced at 24 than at 48 hpi.

Artesunate reduces BKV genome replication. To study the effect of artesunate on BKV genome replication in RPTECs starting at about 36 hpi (12), we measured intracellular BKV DNA and cellular ACY loads in untreated and artesunate-treated RPTECs by qPCR at 24 and 48 hpi and expressed the results as the number of GEq per cell. At 24 hpi, the intracellular BKV DNA measured mainly represents input genomes. In agreement with this, no significant difference in the intracellular BKV DNA load was found between untreated and artesunate-treated cells (Fig. 2D). From 24 to 48 hpi, the intracellular BKV DNA load in untreated cells was found to increase by 0.7 log. However, in artesunate-treated cells, the increase was only 0.1 log. On the basis of this, artesunate was found to reduce the replication of BKV DNA by 73% compared to untreated and late protein expression. Cell extracts were made from untreated and artesunate-treated BKV-infected RPTECs at 24 and 48 hpi, and Western blotting was performed with polyclonal rabbit anti-N-terminal LT-ag, anti-VP1, and anti-agnoprotein (Agno) serum and a monoclonal antibody directed against the housekeeping protein GAPDH. The anti-N-terminal LT-ag serum recognizes LT-ag and sT-ag. Lane MW, molecular weight markers (indicated on the right, in thousands). (D) BKV genome replication. Untreated and artesunate-treated BKV-infected RPTECs were harvested at 24 and 48 hpi, DNA was extracted, and qPCR for BKV and the cellular gene ACY was performed. Intracellular BKV DNA was expressed as number of GEq per cell. Mean values of the number of GEq/cell ± SDs of four experiments (each of two experiments was performed in two wells) are presented.
Artesunate Inhibits Polyomavirus BK Replication

FIG 3 Effect of increasing concentrations of artesunate on viability of mock- and BKV-infected RPTECs. (A) Real-time cell proliferation. RPTECs were seeded in an E plate 16, and 25 h later, half of the medium in the wells was replaced by growth medium with artesunate to reach the indicated concentrations (top). In some wells, purified BKV Dunlop was also added (bottom). The CI, a combined measure of cell adhesion, proliferation, and size, was monitored from the time of seeding until 96 h posttreatment and infection by an xCELLigence RTCA DP instrument. The CIs, normalized to the value for the cell-free background, are shown as mean values ± SDs of 2 wells. The results of one representative experiment out of two are shown. (B) DNA replication and metabolic activity. Cellular DNA replication (BrdU) and the total metabolic activity (resazurin) of artesunate-treated mock- and BKV-infected RPTECs were measured at 72 hpi (i.e., 70 h posttreatment). Mean values of the percent absorbance of untreated cells ± SDs of three experiments (each experiment was performed in three wells) are presented. (C) Cytotoxicity. BKV-infected RPTECs were treated with the indicated concentrations of artesunate from 0.3 to 80 µM on cellular DNA replication (measured by the BrdU assay) and on total metabolic activity (measured by the resazurin assay) at 72 hpi. As before, artesunate was added at 2 hpi and left on the cells until 72 hpi (i.e., 70 h posttreatment), when measurements were performed. Artesunate was found to reduce both cellular DNA replication and total metabolic activity in a concentration-dependent manner. The DNA replication in mock- and BKV-infected cells was reduced between 0 and 90% from about 7 h after addition. However, the inhibition was partly reversed during observation for 96 h posttreatment. Also, for artesunate at 5 and 10 µM, a partial reversal of inhibition was seen, though to a lesser extent than with the lower concentrations. For artesunate at 20 and 40 µM, no reversal of inhibition was seen during observation for 96 h posttreatment. The DMSO control did not seem to affect proliferation. In summary, addition of artesunate completely stopped the proliferation of both mock-infected and BKV-infected RPTECs from about 7 h after addition. However, the inhibition was partly reversed after about 40 h for concentrations up to 10 µM but not for higher concentrations.

We next examined the effect of artesunate at concentrations ranging from 0.3 to 80 µM on cellular DNA replication (measured by the BrdU assay) and on total metabolic activity (measured by the resazurin assay) at 72 hpi. As before, artesunate was added at 2 hpi and left on the cells until 72 hpi (i.e., 70 h posttreatment), when measurements were performed. Artesunate was found to reduce both cellular DNA replication and total metabolic activity in a concentration-dependent manner. The DNA replication in mock- and BKV-infected cells was reduced between 0 and 90% (Fig. 3B). Of note, the DNA replication was slightly more affected in mock-infected cells than in infected cells. For instance, artesunate at 10 µM reduced cellular DNA replication in mock- and BKV-infected cells by 82 and 68%, respectively. The DMSO control reduced cellular DNA replication in mock- and BKV-infected cells by 16 and 24%, respectively.

The total metabolic activity in mock- and BKV-infected cells the level of replication for untreated cells (Fig. 2D). Of note, the cellular ACy load was 17% lower in artesunate-treated cells than untreated cells at 48 hpi, probably reflecting the lower cell numbers. The DMSO control decreased BKV replication by 9%. We conclude that artesunate inhibits BKV genome replication in RPTECs.

Artesunate induces cytostatic effects in RPTECs. Although phase-contrast microscopy of mock- and BKV-infected cells exposed to artesunate at a concentration up to 10 µM for 70 h (72 hpi) did not reveal changes in morphology compared to the morphology of untreated cells, the nuclear staining (blue) unveiled a reduction in cell numbers when more than 1.25 µM artesunate was used (Fig. 1B). We therefore decided to investigate the effect of artesunate on the viability of mock- and BKV-infected RPTECs in more detail. First, we examined the influence of artesunate concentrations ranging from 1.25 to 40 µM in real time for 96 h using the xCELLigence system. In order to keep the disturbance of the measurements to a minimum, artesunate was added together with the virus. Artesunate influenced the cell index (CI) in a similar manner in both mock- and BKV-infected cells (Fig. 3A). All concentrations tested completely stopped cell proliferation. With artesunate at 40 µM, cell proliferation stopped at 7 h posttreatment, while this effect was delayed up to 17 h posttreatment when lower concentrations were used. For artesunate at 1.25 and 2.5 µM, the inhibition of proliferation was partly abolished at 48 h posttreatment. Also, for artesunate at 5 and 10 µM, a partial reversal of inhibition was seen, though to a lesser extent than with the lower concentrations. For artesunate at 20 and 40 µM, no reversal of inhibition was seen during observation for 96 h posttreatment. The DMSO control did not seem to affect proliferation. In summary, addition of artesunate completely stopped the proliferation of both mock-infected and BKV-infected RPTECs from about 7 h after addition. However, the inhibition was partly reversed after about 40 h for concentrations up to 10 µM but not for higher concentrations.

The total metabolic activity in mock- and BKV-infected cells...
was reduced by between 0 and 85% (Fig. 3B). While mock-infected cells were slightly more affected by concentrations below 10 μM, infected cells were most affected by concentrations above 10 μM. Artesunate at 10 μM reduced total metabolic activity in both mock- and BKV-infected cells by approximately 50%. The DMSO control gave no reduction in total metabolic activity.

After having shown that artesunate leads to significantly deceased cell numbers (Fig. 1B), cellular DNA replication (Fig. 3B), total metabolic activity (Fig. 3B), and cell proliferation (Fig. 3A), we investigated whether artesunate also induced cytotoxic effects by measuring the LDH activity in supernatants. Since the half-life of released LDH has been found to be only 9 h in cell culture (39), we decided to measure LDH at 24, 48, and 72 hpi. The results are presented as percent cytotoxicity, where 100% cytotoxicity was the total LDH level measured in supernatants after complete lysis of the cells. In untreated cells, a cytotoxicity of 6% was found at 24 hpi, and presumably due to viral cytopathic effects, it increased up to 16% at 72 hpi. In artesunate-treated cells, concentrations from 0.6 to 10 μM induced slightly higher relative cytotoxicity at 24 and 48 hpi, while no difference from untreated cells was observed at 72 hpi (Fig. 3C). Artesunate at 40 μM, on the other hand, increased the cytotoxicity at every time point investigated. At 48 hpi the cytotoxicity was 4 times higher than in untreated cells and it increased to a maximum of 26% at 72 hpi. The DMSO control did not affect the cytotoxicity. In summary, artesunate concentrations up to 10 μM gave only a weakly increased cytotoxic effect at 24 and 48 hpi, while 40 μM increased the cytotoxicity at all time points but especially at 48 hpi.

The finding of a mainly cytostatic effect of artesunate made us ask how the cell cycle distribution in RPTECs was affected by artesunate. We decided to investigate the effect of 10 and 40 μM artesunate after 24 and 72 h of treatment by performing flow cytometry of ethanol-fixed and Draq5-stained mock-infected RPTECs. After 24 h of treatment with artesunate at 10 μM, the cell population in S phase was decreased by 42% compared to the S-phase population of untreated cells. At the same time, the cell population in G2/M phase was increased 5% and that in G0/G1 phase was increased almost 2% (Fig. 4A and B). Artesunate at 40 μM gave exactly the same decrease of the cell population in S phase, but here the cell population in G0/G1 phase was decreased by 17%, while the cell population in G2/M phase was increased by 56% (Fig. 4A and B). At 72 h posttreatment, artemesunate at both 10 and 40 μM decreased the cell population in S phase by approximately 50%, while the cell population in G2/M phase was increased by 9 and 132% at concentrations of 10 and 40 μM, respectively (Fig. 4C). In summary, artesunate dramatically decreased the cell population in G0 phase, but it appeared that the different concentrations of artesunate did this by arresting the cells at different points in the cell cycle.

To study the effect of artesunate on the cell cycle distribution on the single-cell level, the Click-iT EdU cell proliferation assay was used. This assay allowed us to detect cells with DNA replication during the time span when EdU was present. First, artesunate at 10 μM or 40 μM was added to mock-infected RPTECs, and then, 22 h later, EdU was added for 2 h before the cells were fixed at 24 h posttreatment. In agreement with our flow cytometry data, a 24-h artesunate exposure resulted in an approximate halving of the number of cells going through S phase independent of the artesunate concentration used (Fig. 4D). Similar results were also obtained after 72 h of artesunate exposure (data not shown). In summary, artemesunate decreased the cell population in S phase.

We conclude that artesunate has a rapid and strong cytostatic effect on both mock- and BKV-infected RPTECs. For artesunate concentrations of 10 μM or less, inhibition is short-lived and seems to be caused by cell cycle arrest in G0. However, for higher artesunate concentrations, the inhibition is longer lasting and seems to also involve G2 arrest.

The cytostatic and the antiviral effects of artesunate in BKV-infected RPTECs are reversible. The data demonstrate that artesunate at 10 μM inhibits BKV replication and host cell proliferation without overt cytotoxicity. In order to assess whether these effects of artesunate may be reversed by removal of artesunate, mock- and BKV-infected cells were treated with artesunate at 10 μM for 10 h before the artesunate was removed, the cells were washed, and medium without artesunate was added. A 10-h treatment was chosen since adverse effects on cell proliferation had been found from approximately 7 h posttreatment. Subsequently, the BKV load was measured at 72 hpi, cell proliferation was monitored in real time until 72 hpi (i.e., 72 h posttreatment), and the cell cycle distribution was analyzed by flow cytometry at 72 h posttreatment. By interrupting the treatment after 10 h, the BKV DNA load was reduced by only 0.1 log unit, whereas it was decreased by 0.6 log after 70 h of treatment (i.e., a 24% versus a 75% reduction) (Fig. 5A). The real-time viability assay showed that BKV-infected cells (Fig. 5B) and mock-infected cells (data not shown) almost returned to normal proliferation approximately 3 h after artesunate was removed. In agreement with this, the flow cytometry data showed that the cell cycle distribution 62 h after the removal of artesunate was similar to that of untreated cells (Fig. 5C).

We conclude that the strong cytostatic effects of artesunate on RPTECs can be almost completely reversed by removal of artesunate. However, by restoring the proliferation potential of the cells, most of the antiviral activity of artesunate was lost.

The SI of artesunate in RPTECs is low, likely reflecting the dependence of BKV on host cell proliferation. The relative efficacy of an antiviral drug may be defined by the selectivity index (SI), usually expressed as SI50 (CC50/EC50). To find the EC50, we calculated the 50% reduction in the BKV DNA load at 72 hpi on the basis of the data in Fig. 1A, and this was found to be 4.2 μM (Fig. 6A). Since the cytotoxic effects of artesunate found here were far less than 50%, we decided to determine the CC50 on the basis of cellular DNA replication, as previously described for CMX001 (31), ofloxacin, and levofloxacins (32). The CC50 was therefore calculated on the basis of the BrdU incorporation data in Fig. 3A, and this was found to be 2.4 μM (Fig. 6B). These results suggested an SI50 (CC50/EC50) of about 0.6 for artesunate on RPTECs. We conclude that the selectivity index determined for proliferating RPTECs was low; however, the present calculation was based on a method much more sensitive than that usually employed for cell toxicity.

**DISCUSSION**

An antiviral treatment option for PyVAN is urgently needed, but drugs efficiently inhibiting BKV replication are lacking. We have explored the antiviral activity of artesunate, an antimalarial drug recently shown to have antiviral activity, on BKV replication in human primary renal proximal tubular epithelial cells. The data presented here indicate that artesunate inhibits BKV replication in...
In a productive BKV infection, transcription of early pre-mRNA is the first step taking place after uncoating of the BKV genome. This is followed by splicing to 3 functional early mRNAs encoding LT-ag, st-ag, and truncated LT-ag. Our detailed study with 10 µM artesunate demonstrated a 30% reduction of normal-LT-ag mRNA levels at 24 hpi. In agreement with this, an approximately 75% reduction of both LT-ag and st-ag protein expression was found by Western blotting at 24 hpi. Of note, this finding is different from the finding in our previous studies with cidofovir and CMX001, where no reduced LT-ag mRNA and protein expression was found as early as 24 hpi (12, 31). Since LT-ag functions as the replicative helicase and initiator of viral DNA replication by melting the double-stranded DNA strand to initiate viral effect of artesunate on BKV replication in RPTECs is closely related to its strong but partly reversible cytostatic effect.

A concentration-dependent manner. With artesunate at 10 µM, a 65% reduction of the extracellular BKV DNA load at 72 hpi was found, reflecting a significant decrease in infectious progeny release. Several steps in the BKV replication cycle were inhibited, such as early BKV mRNA and protein expression, as well as BKV genome replication and late mRNA and protein expression. In addition, artesunate inhibited BKV proliferation in a concentration-dependent manner. With artesunate at 10 µM, cellular DNA replication was reduced 68%, while metabolic activity was reduced 50%. In mock-infected cells, the reduction in DNA replication was 82%, while metabolic activity was the same as that for infected cells. By real-time monitoring of cell proliferation, a reduction was registered as early as 7 h after artesunate addition. The effect was mainly cytostatic and was transient when concentrations up to 10 µM were used. The mechanism seems to involve a concentration-dependent arrest in G₀ or G₂, reducing the cell population in S phase. Removal of the drug almost completely reversed the cytostatic effects but also reduced the inhibition of BKV replication. Taken together, these data suggest that the antiviral effect of artesunate on BKV replication in RPTECs is closely related to its strong but partly reversible cytostatic effect.
fork unwinding and by recruiting cellular enzymes like DNA polymerase α primase, topoisomerase I, and replication protein A (40, 41), the low LT-ag level detected could be responsible for the 73% reduced viral DNA replication observed in artesunate-treated cells at 48 hpi. However, part of the inhibition was probably caused by other mechanisms, like cell cycle arrest (see below), since cellular DNA replication, which is completely independent of LT-ag, was also reduced.

In addition to the initiation and coordination of DNA replication, LT-ag stimulates late gene expression from newly replicated BKV DNA by activating transcription from the late promoter (42). At 48 hpi, a 47% reduction in VP1 mRNA transcripts and corresponding 64% and 49% reductions of VP1 and agnoprotein, respectively, were found. Both the lack of LT-ag and the reduced BKV genome replication, with the latter leading to fewer templates for late transcription, probably contributed to the reduced late mRNA and protein expression. We cannot exclude the possibility of an additional direct inhibitory effect on late transcription and translation. Not unexpectedly, the inhibition of all investi-
gated steps of the BKV replication cycle culminated in a 65% reduction of the BKV DNA load and a corresponding reduction of infectious progeny. The reason that some cells expressed both early and late BKV proteins and therefore seemed to be resistant to even high artesunate concentrations is unknown.

With regard to the effect on host cells, the RPTECs, artesunate induced a significant concentration-dependent inhibition of cell proliferation. The effect was already apparent 7 to 17 h after the start of treatment, when artesunate concentrations from 1.25 μM completely stopped cell proliferation. Both cellular DNA replication and metabolic activity were significantly reduced, but the LDH assay revealed that the effect was cytostatic and not cytotoxic. The cytostatic effect was characterized in more detail by flow cytometry analysis. Artesunate decreased the numbers of cells in S phase by more than 50%. However, while artesunate at 40 μM increased the numbers of cells in G2/M phase, artesunate at 10 μM slightly increased the numbers of cells in G0/G1 phase. The results therefore suggest that the mechanism inhibiting cell proliferation depends on the artesunate concentration. This is also supported by our real-time proliferation analysis, where only cells treated with artesunate at concentrations of 10 μM or less, which were possibly arrested in G0 phase, spontaneously reinitiated proliferation after 40 to 50 h. Of note, artesunate and dihydroartemisinin have previously been described to decrease the numbers of cells in S phase in A431 human epidermoid carcinoma cells (43), cells of the human leukemia cell line K562 (44), and cells of the murine myeloma cell line SP2/0 (45). Interestingly, while some found simultaneously increased numbers of cells in G2/M phase (43) (45), others described increased numbers of cells in G2/M phase (46). Apparently, artesunate also induced apoptosis in many cancer cell lines (43, 45, 46). Since we did not detect any sub-G1-phase fraction when performing flow cytometry, we concluded that artesunate at 10 and 40 μM does not induce apoptosis in RPTECs.

Interestingly, the reduction of cellular DNA replication was more pronounced in mock-infected than in BKV-infected RPTECs; i.e., artesunate at 10 μM reduced cellular DNA replication in mock- and BKV-infected cells by 82 and 68%, respectively. One explanation for this could be that the BKV infection counteracted the inhibitory effect of artesunate. It is well-known that polyomaviruses can override the cell cycle, as recently also demonstrated for BKV (47). The mechanism has been studied in detail for simian virus 40 (SV40) (41). Early in infection, LT-ag interacts with retinoblastoma (Rb) proteins to free the transcription factor E2F. This drives the infected cells into the beneficial replicative S phase, which induces a DNA damage response. However, LT-ag binds and inactivates p53, thereby preventing apoptosis or cellular senescence. In agreement with this, we have previously demonstrated that BKV infection increases cellular DNA replication in RPTECs (12).

In our study, cultured cells infected with BKV received a single dose of artesunate, and this gave a transient effect, at least at concentrations up to 10 μM. It is possible that repetitive smaller doses would have given a stronger effect, as seen in an in vitro study of CMV (48). For clinical use, frequent dosing would probably be necessary, since the drug is rapidly metabolized in the liver and has a half-life of only 0.36 or 2.14 h, depending on the route of administration (reviewed in reference 29). Due to the short half-life, the current World Health Organization-recommended treatment of malaria is artesunate in combination with other antimalaria drugs, and both drugs are usually administered for 3 to 7 days (49). However, a 7-day treatment regime would probably not be sufficient for a successful treatment of PyVAN or PyVHC. Mathematical modeling has shown that a >90% reduction of renal BKV replication must be maintained for up to 10 weeks in order to clear plasma and urine viral loads (3). Long-term treatment might become a problem, since both animal and human studies suggest that long-term rather than short-term peak concentrations of artesunate may cause toxicity (reviewed in reference 50).

Concerning the present study, only minimal cytotoxic effects were found following 72 h of artesunate treatment, but significant cytostatic effects were revealed as early as 7 h after treatment start. Interestingly, cytostatic effects have not been reported in other antiviral studies on artesunate. There may be several reasons for this, for instance, the use of different cells or the treatment of fully confluent cells, but it is also possible that mainly cytotoxic effects were investigated. For instance, the effect on host cells was investigated by measuring residual cell layers by the LDH assay at 4 or 7 days after artesunate addition to confluent cells (17, 51) or by measuring the LDH release in the supernatant 7 days after artesunate addition (18). Although short-term artesunate treatment is considered safe, there have been some reports on hemolytic anemia after treatment of severe malaria, but this may actually be a consequence of the severe malaria and not of artesunate (52). Even though the cytostatic effects will probably not affect the slowly cycling epithelial cells of healthy kidneys or the urinary bladder, it may jeopardize the regeneration of epithelium damaged by PyVAN or PyVHC.

In order to kill the malaria parasites, only nanomolar concentrations of artesimins are needed (reviewed in reference 50). However, to inhibit the replication of BKV in RPTECs by 50% (i.e., the EC50), a concentration of 4.2 μM (1.6 μg/ml) artesunate was needed, and it is still unclear if this concentration can be attained in kidney epithelial cells in vivo. Following intravenous administration of artesunate in healthy volunteers, plasma concentrations up to 217 μM (83.34 μg/ml) have been described (29, 53, 54), indicating that the EC50 could at least be achieved in the plasma.

Adverse side effects of drugs may be acceptable if they occur at concentrations higher than the concentration required to achieve the therapeutic effect. We calculated a SI50 of 0.6. Since the EC50 was found to be in the same range as that reported for herpesviruses (1.5 to 6.4 μM) (24, 25, 48, 51), the low SI50 mainly resulted from the low CC50 of only 2.4 μM. However, unlike most researchers, we calculate the CC50 using the very sensitive BrdU assay. Nevertheless, comparing it to the SI50 of ofloxacin (6.48) and levofloxacin (7.13), which were obtained using the same assay and cells (32), the SI50 of artesunate was found to be at least 10 times lower.

In conclusion, artesunate inhibits BKV replication in RPTECs in a concentration-dependent manner by impairing BKV gene expression and genome replication. Notably, the antiviral effect is closely connected to the cytostatic effect of the drug on the host cell. Importantly, both BKV inhibition and cytostatic effects are reversible. Whether artesunate will be useful for the treatment of BKV disease can be answered only by performing carefully designed clinical studies also addressing long-term effects. Such studies need to take the short half-life of artesunate into account and might consider combination therapy which is also indicated when using artesunate in the treatment of malaria.
REFERENCES


Artesunate Inhibits Polyomavirus BK Replication


