Investigation of the antiviral effects of artesunate on BK and JC polyomavirus replication

Biswa Nath Sharma
A dissertation for the degree of Philosophiae Doctor – July 2014
Cover photo: BKPyV infected primary human urothelial cells. 
(adapted from Li et al., 2013. Characteristics of polyomavirus BK (BKPyV) infection in primary human urothelial cells. Virology 440, 41-50)
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By

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A dissertation for the degree of Philosophiae Doctor – July 2014

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Acknowledgement

This study was conducted at the Department of Microbiology and Infection Control at the University Hospital of North Norway from 2011 to 2014. The study was financially supported by the Northern Norway Regional Health Authority Medical Research Program.

First of all, I would like to express my sincere gratitude to my supervisor, Dr. Christine Hanssen Rinaldo for sharing her immense knowledge, continuous support, motivation and guidance throughout my PhD study. In fact, this study would not have been possible without her kind guidance and support. THANK YOU VERY MUCH!

In the Department of Microbiology and Infection Control, I would like to thank:

- Tore Guttleberg for being my co-supervisor
- Gunnar Skov Simonsen and Kristin Helene Hauan for their unconditioned continuous support
- Garth D Tylden for critical reading of the manuscript, valuable comments and inputs and interesting discussions in our office and friendship
- Stian Henriksen for excellent technical support, critical reading of the manuscript, valuable comments and inputs and friendship
- all the past and present members for making a nice working environment

I would also like to thank Ruomei Li, now at UiT The Arctic University of Norway for fruitful collaboration, and Hans H Hirsch, University of Basel, for continuing education in the journal club and valuable input to my thesis.

Thanks to my parents, sisters and friends as well for their continuous support.

The last but not the least, I would like to thank my dearest wife Bibhash for love, constant encouragement and understanding; and my son Baibhab for his joyful distraction. I know both of you sacrificed a lot during my study period.

Biswa Nath Sharma
Tromsø, Norway,
July, 2014
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Paper IV
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Antiviral Effects of Artesunate on JC Polyomavirus Replication in COS-7 cells. Submitted Manuscript

Appendix I

Appendix II
Abbreviations

µM microMolar
ATM Ataxia Telangiectasia Mutated
ATR Ataxia Telangiectasia and Rad3-related
BKPyV BK Polyomavirus
bp base pair
CNS Central nervous system
CSF Cerebrospinal fluid
DMSO Dimethyl Sulfoxide
DNA deoxyribonucleic acid
GEq Genome Equivalent
HCMV Human cytomegalovirus
HHV-6B Human Herpes Virus-6B
hpi hours postinfection
hpt hours posttreatment
HPyV 6 Human Polyomavirus 6
HPyV 7 Human Polyomavirus 7
HSCT hematopoietic stem cell transplant
HSV-2 Herpes Simplex Virus-2
HUCs Human Urothelial Cells
JCPyV JC Polyomavirus
KIPyV KI Polyomavirus
KT Kidney Transplant
LTag Large T antigen
MCPyV Merkel cell Polyomavirus
miRNAs microRNAs
MPyV Murine Polyomavirus
NCCR Non-Coding Control Region
PCR Polymerase Chain Reaction
PML Progressive Multifocal Leukoencephalopathy
Pol α DNA Polymerase α
pRB  protein Retinoblastoma
PyVAN  Polyomavirus-associated Nephropathy
PyVHC  Polyomavirus-associated Hemorrhagic Cystitis
RPTECs  Renal Proximal Tubular Epithelial Cells
SOT  Solid Organ Transplant
sTag  small T antigen
SV40  Simian Virus 40
TruncTag  Truncated T antigen
TSPyV  Trichodysplasia Spinulosa-associated Polyomavirus
WUPyV  WU Polyomavirus
Human Polyomavirus

Discovery

Polyomaviruses are widely distributed in vertebrates. The era of polyomavirus began with the accidental discovery of murine polyomavirus (MPyV) in 1958 followed by the discovery of simian virus 40 (SV40) in 1960 in monkey kidney cells that were used to prepare the polio vaccine (267, 270). The name polyomavirus is derived from the Greek words *poly*: many and *oma*: tumor following the observation that tissue extracts containing MPyV caused the development of multiple tumors when injected into newborn mice. A decade later, the two human viruses JC polyomavirus (JCPyV) and BK polyomavirus (BKPyV) were independently discovered in 1971 (100, 219). BKPyV was isolated from the urine of a kidney transplant (KT) patient with the initials B.K. suffering from ureteric stenosis while JCPyV was isolated from the brain of a patient with the initials J.C. who died of progressive multifocal leukoencephalopathy (PML). Both viruses were isolated using cell culture and visualized by electron microscopy, a novel technology at that time. It took approximately 40 years for the next discovery of human polyomaviruses. In 2007, KI polyomavirus (KIPyV) and WU polyomavirus (WUPyV) were isolated independently from the nasopharyngeal aspirates of patients presenting with respiratory infections (9, 103). While KIPyV was identified by cloning of DNase protected deoxyribonucleic acid (DNA) followed by random polymerase chain reaction (PCR) and conventional sequencing, WUPyV was identified by generation of a random library followed by high throughput DNA sequencing. The names KI and WU were derived from the initials of the institutes where the viruses were discovered. Since then, in just 6 years, 8 more human polyomaviruses were described. This sudden increase in the number of human polyomaviruses was due to the increased awareness and advances in molecular diagnostic technology. In 2008, Merkel cell polyomavirus (MCPyV) was isolated from
merkel cell cancer tissue by digital transcriptome subtraction (90). In 2010, Human polyomavirus 6 and 7 (HPyV6 and HPyV7) were detected by amplification of circular DNA from skin swabs from healthy individuals using random hexamer-primed rolling circle amplification and followed by cloning and sequencing (249). In the same year, trichodysplasia spinulosa-associated polyomavirus (TSPyV) was detected by rolling circle amplification, cloning and sequencing of circular DNA from the spicules of a immunosuppressed heart transplant patient with the rare skin disease trichodysplasia spinulosa (279). In 2010, Human polyomavirus 9 (HPyV9) was discovered in the serum of a KT patient by the use of a degenerated PCR targeting the VP1 gene (250). Human polyomavirus 10 (HPyV10) was discovered by rolling circle amplification, followed by cloning and sequencing of DNA from some condylomas from a patient with a rare skin disorder (47). The two variants of this virus, Malawi polyomavirus (MWPyV) and MX polyomavirus (MXPyV) were discovered in stool specimens of children suffering from diarrhea by performing shotgun pyrosequencing of purified viral particles or unbiased deep sequencing of cDNA libraries, respectively (262, 292). Saint Louis polyomavirus (STLPyV) was also discovered in a stool specimen of a child with diarrhea by the use of rolling circle amplification followed by pyrosequencing (179). Recently, Human polyomavirus 12 (HPyV12) was detected by generic PCR of DNA from liver samples of patients who underwent surgery, followed by nested long-distance PCR and sequencing (166). Very recently, New Jersey polyomavirus (NJPyV-2013) was detected in endothelial cells from a muscle biopsy of a pancreas-transplant patient suffering from retinal blindness and vasculitic myopathy by the use of high-throughput nucleic acid sequencing (197). So far, 13 different human polyomaviruses have been described. Even though all the human polyomaviruses except HPyV6 and HPyV7 were discovered in the disease context, a
causal role for disease has only been established for BKPyV, JCPyV, MCPyV and TSPyV (62).

**Classification**

The classification of polyomavirus has changed in recent years. In the first classification, polyomavirus was classified within the *Papovaviridae* family together with papillomavirus. In 2000, the International Committee on Taxonomy of Viruses split the *Papovaviridae* family into the two families *Polyomaviridae* and *Papillomaviridae*. *Polyomaviridae* consists of only one genus: *polyomavirus*. Due to the increasing number of polyomaviruses and their biological and genomic differences, in 2007 the International Committee on Taxonomy of Viruses formed the *Polyomaviridae* Study Group to revise the classification again. Accordingly, in 2010 the *Polyomaviridae* Study Group recommended a reclassification based on the host range, genetic repertoire and DNA sequence identity over the whole genome (150). This new classification would divide the current single genus into two mammalian genera (*Orthopolyomavirus* and *Wukipolyomavirus*) and one avian genus (*Avipolyomavirus*). Interestingly, all the polyomavirus known to cause diseases would belong to the genus *orthopolyomavirus*. However, the suggested classification was still not included in the last virus taxonomy release in 2013.
**BK and JC polyomavirus**

**Virion Structure**

Polyomaviruses are morphologically indistinguishable and share common structural characteristics. They are non-enveloped and small, measuring approximately 40-45 nm in diameter. The capsid is composed of 72 pentameric VP1 capsomers arranged in icosahedral symmetry (T=7) and encloses the viral genome (Figure 1). Each capsomer under its surface contains one molecule of either VP2 or VP3. The virion consists of 88% protein and 12% DNA (6).


**Figure 1. Polyomavirus Virion.** Schematic illustration of polyomavirus virion showing the organization of capsid proteins enclosing double stranded DNA genome wrapped around histones (ViralZone: www.expasy.org/viralzone, Swiss Institute of Bioinformatics).

**Genome**

The BKPyV and JCPyV genomes consist of a single copy of circular, double stranded DNA of approximately 5.2 kilo base pairs (bp) with a molecular weight of approximately $3.2 \times 10^6$ Dalton (6). Inside the capsid, the genome is associated with cellular histones to form about 21 nucleosomes like a minichromosome (58, 193). Each nucleosome contains two copies of the four different cellular histones H2A, H2B, H3 and H4. The coding capacity of the rather small genome is extended by the
use of overlapping open reading frames and frame shifts and by the use of internal translation initiation codons. There is approximately 75% sequence identity between the genomes of BKPyV and JCPyV (257).

Figure 2. Schematic illustration of the circular double stranded DNA genome of archetype BKPyV WW (Gen Bank AB211371.1) (A) (modified from (234)) and of archetype JCPyV CY (Gene Bank AB038249) (B). The common genome organization shows the early- and late viral gene region separated by the regulatory non-coding control region (NCCR) containing numerous transcription factor binding sites. Arrow indicates the direction of transcription of the gene and the dash (-) indicates a pre-microRNA (miRNA) that generates two miRNAs. The NCCR of BKPyV is divided into 5 different sequence blocks denoted O, P, Q, R and S where the O block contains the origin of replication. The NCCR of JCPyV is divided into 6 blocks denoted A, B, C, D, E and F in addition to Ori block containing the origin of replication. The length in base pairs is indicated within each NCCR block.
Like other polyomaviruses, the BKPyV (Figure 2A) and JCPyV (Figure 2B) genomes are functionally divided into 3 different regions: an early viral gene region, a late viral gene region and a non-coding control region (NCCR).

The early viral gene region is located upstream of the origin of replication. The early viral gene region encodes early proteins Large Tumor antigen (LTag) and small Tumor antigen (sTag). In addition, the early viral gene region of BKPyV encodes a truncated T antigen (TruncTag) while that of JCPyV encodes T'135, T'136 and T'165 antigens (1, 272). This means that BKPyV and JCPyV express 3 and 5 different early proteins, respectively. All these proteins are translated from mRNAs produced by alternative splicing of a single, large transcript (pre-mRNA).

The late viral gene region on the downstream side of the origin of replication encodes the late proteins VP1, VP2, VP3 and agnoprotein. So far, BKPyV and JCPyV are the only human polyomaviruses encoding agnoprotein. Like early proteins, the late proteins are also translated from an alternatively spliced pre-mRNA. Transcription of the late region occurs in the opposite direction and from the strand complementary to that used for early transcription. VP2 and VP3 are translated from alternative start codons on a common mRNA transcript in the same reading frame while VP1 and agnoprotein are translated in a different reading frame (145, 154).

The NCCR is located between the translation start codons for the early genes and late genes and is a nucleosome free region of the genome (234). The NCCR typically contains the origin of replication, the binding sites for LTag, promoter and transcription enhancer elements and thus, contains the regulatory information for transcription of the genes (198). The specific host cell tropism is also at least in part defined by the NCCR (15). The most commonly found strains in urine of healthy
people, BKPyV WW and JCPyV CY contain the archetype NCCR and are considered to be the transmissible strains (67, 85, 111, 117). The archetype NCCR of BKPyV WW has been arbitrarily divided into five sequence blocks, O (142bp), P (68bp), Q (39bp), R (63bp) and S (63bp) (Figure 2A) where the origin of replication is located in the O block. Similarly, the archetype NCCR of JCPyV CY is arbitrarily divided into six blocks A (36bp), B (23bp), C (55bp), D (66bp), E (18bp) and F (69bp) in addition to Ori (117bp) containing the origin of replication (Figure 2B) (188, 198). Deletion, duplication and rearrangement of the NCCR blocks may occur during replication and the NCCR is then referred to as a rearranged NCCR. The rearranged NCCR has often been linked with a high replication capacity \textit{in vitro} and \textit{in vivo} and therefore also with disease (110, 111, 213).

**Viral proteins**

**Table 1.** An overview of viral proteins of BKPyV Dunlop (GenBank V01108) and JCPyV CY (GenBank AB038249) and their % identity (modified from (6, 72, 144))

<table>
<thead>
<tr>
<th>Viral Proteins</th>
<th>Cellular localization</th>
<th>Number of aminoacids BKPyV/JCPyV</th>
<th>Mol. Wt. (kDa) BKPyV/JCPyV</th>
<th>% identity BKPyV/JCPyV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Early</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LTag</td>
<td>Nucleus</td>
<td>695/688</td>
<td>80.5/79.3</td>
<td>83%</td>
</tr>
<tr>
<td>sTag</td>
<td>Nucleus/Cytoplasm</td>
<td>172/172</td>
<td>20.5/20.2</td>
<td>78%</td>
</tr>
<tr>
<td>TruncTag</td>
<td>Nucleus</td>
<td>135/-</td>
<td>17/-</td>
<td></td>
</tr>
<tr>
<td>T' 165 antigen</td>
<td>Nucleus</td>
<td>/165</td>
<td>-/NA</td>
<td></td>
</tr>
<tr>
<td>T' 136 antigen</td>
<td>Nucleus</td>
<td>/136</td>
<td>-/NA</td>
<td></td>
</tr>
<tr>
<td>T' 135 antigen</td>
<td>Nucleus</td>
<td>/135</td>
<td>-/NA</td>
<td></td>
</tr>
<tr>
<td><strong>Late</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VP1</td>
<td>Nucleus</td>
<td>362/354</td>
<td>40.1/39.6</td>
<td>63%</td>
</tr>
<tr>
<td>VP2</td>
<td>Nucleus</td>
<td>351/344</td>
<td>38.3/37.4</td>
<td>80%</td>
</tr>
<tr>
<td>VP3</td>
<td>Nucleus</td>
<td>232/225</td>
<td>26.7/25.7</td>
<td>77%</td>
</tr>
<tr>
<td>Agnoprotein</td>
<td>Cytoplasm/Perinuclear</td>
<td>66/71</td>
<td>7.4/8.1</td>
<td>63%</td>
</tr>
</tbody>
</table>

Dash (-): not detected; NA: Not available
**Large T antigen**: LTag which is the major regulatory protein localizes to the nucleus and has multiple functions in the viral replication cycle including initiation of DNA replication, the translation of late genes and autoregulation of early genes. In addition, LTag regulates the cell cycle. Most of the knowledge of LTag functions is derived from SV40 LTag studies. Since SV40 LTag shares more than 70% aminoacid identity to LTags of BKPyV and JCPyV, this suggests the common LTag functions (Table 1) (6). **Figure 3** illustrates the functional domains and major binding sites of SV40 LTag for cellular proteins: such as the N-terminal J domain, responsible for binding with heat shock protein 70 and with DNA polymerase α (Pol α) important for viral DNA replication; the protein retinoblastoma (pRB) binding domain with the LXCXE motif, responsible for binding with the member proteins of pRB family; the Ori binding domain, responsible for binding with the origin of replication in NCCR and with a binding site for topoisomerase I, required for relaxation and reannealing of DNA strands; the Helicase domain, responsible for unwinding of double stranded DNA during replication and with a binding site for Pol α; the Zn finger region, responsible for oligomerization of LTag; the bipartite p53 binding domain, responsible for binding with p53; the ATPase domain, responsible for ATPase activity of LTag; the host range domain, responsible for the host range function and virion assembly; and the Nuclear Localization Signal, responsible for the nuclear translocation of LTag (58, 199, 222, 268).
The organization of functional domains and binding sites for the host cell proteins are shown such as J domain, LXCXE motif of pRB binding domain, Nuclear Localization Signal (NLS), Ori binding domain, Zn finger region (Zn), Helicase domain, bipartite p53 binding domain, ATPase domain, and the host range (HR) domain and the binding sites for cellular proteins heat shock protein (Hsc70), pRB, p107, p130, p53, DNA polymerase α (Pol α) and Topoisomerase I.

The interactions between LTag and cellular proteins are crucial for creating an intracellular environment conducive to viral replication. I will discuss a few of these interactions in detail here to illustrate this point while the direct involvement with viral genome replication will be discussed under **viral replication**. As mentioned above, SV40-, BKPyV- and JCPyV-LTags interact with pocket proteins of tumor suppressor pRB family (pRB, p107 and p130) and p53 (285). The pocket proteins of the pRB family are the key regulators of the cell cycle. The transcription factors of the E2F family induce expression of cellular genes that are essential to mediate S phase entry. Normally, hypophosphorylated pRB binds and inhibits these transcription factors, while phosphorylated pRB releases the factors. In this way, pRB regulates G1- S phase transition (57). In infected cells, LTag interacts with pRB of pRB-E2F
family transcription factor complex and releases the E2F transcription factors, thereby mediating transition from G1- to S phase. Induction of S phase, the cellular DNA synthesis phase, is very important for viral DNA synthesis. The p53 is also a potent transcriptional activator that induces the expression of a number of cellular genes, some of which mediate DNA repair, cell cycle arrest or apoptosis (7). Under normal conditions, p53 is maintained at a low level by binding with the promoter of the \textit{mdm2} gene and stimulating its transcription. The Mdm2 protein then binds p53, inducing its polyubiquitination and subsequent degradation (7). During cellular stress such as DNA damage, this interaction is perturbed and the level of p53 increases leading to DNA repair, cell cycle arrest and possibly apoptosis. In polyomavirus infected cells, however, LTag interacts directly with p53 and block both its negative feedback loop with Mdm2 and its primary regulatory functions, thus preventing cell cycle arrest and apoptosis (reviewed in (7)).

In addition to the multitude of functions described above, SV40 LTag undergoes many post-translational modifications, such as phosphorylation, glycosylation, ADP-ribosylation, acetylation, adenylation and acylation, possibly further modifying its functions (58, 224).

**Small Tumor antigen:** sTag is also a regulatory protein that localizes to both the nucleus and cytoplasm of the infected cells and shares N-terminal region with LTag (70, 86, 222). The function of sTag is still not resolved in detail but most of the functions were identified from the studies of SV40 sTag. The BKPyV and JCPyV sTags share 71 and 68% amino acid identity with SV40 sTag, respectively (Table 1) (6) and this high aminoacid identity suggests that sTags of these viruses have common functions. Like SV40 sTag, JCPyV sTag was also reported to interact with protein phosphatase 2A, altering its phosphatase activity and thereby activating LTag.
by dephosphorylation and in this way, sTag is indirectly involved in the viral genome replication and also in cell transformation particularly in the setting of low LTag level (36, 220, 222).

**Other T antigens:** The JCPyV T'135, T'136 and T'165, collectively referred to as JCPyV T'ags where the numbers indicate the number of aminoacids, share 132 aminoacids with the N-terminal region of LTag containing the J domain, the pRB binding domain and the nuclear localization signal. These proteins localize to the nucleus and interact with pRB proteins via the LXCXE motif and are probably involved in cellular transformation and viral DNA replication (37, 225). The BKPyV truncTag also shares 133 aminoacids with the N-terminal region of BKPyV LTag and is structurally similar to JCPyV T'ags (1), suggesting that truncTag may have functions similar to those of JCPyV T'ags.

**VP1:** VP1 is the major structural protein and contributes 75% of the total virion protein mass (40). During viral replication, it localizes to the nucleus and forms the capsid or outer shell of the virion. In the capsid, 360 VP1 monomers are arranged in 72 pentamers. The flexible C-terminal end of VP1 connects each pentamer to other pentamers by hydrophobic bonds which are further strengthened by disulfide bonds (58). In addition to being crucial for capsid formation, VP1 is also important for the genome packaging during virus assembly (reviewed in (217)). Moreover, the VP1 contains a variable antigen binding region, which serves as the ligand for viral attachment to host cells (81, 148).

**VP2 and VP3:** VP2 and VP3 are minor structural proteins and also localize to the nucleus of infected cells. In the virion, VP2/VP3 functions as a bridge to connect VP1 and the genome (147). Very little is known about their function. Recently, JCPyV
VP2/VP3 was shown to interact with LTag and increase binding to the origin of replication thereby enhancing the replication of viral DNA (242). In addition, an essential role of VP2/VP3 in the packaging of JCPyV DNA has been suggested (101). BKPyV and JCPyV share 80 and 77% amino acids identity in VP2 and VP3, respectively (Table 1) (6) thus suggesting for similar functions.

**Agnoprotein**: Agnoprotein is a non-structural protein that localizes mainly to the cytoplasm and the perinuclear region although a minor fraction is detectable in the nucleus (149, 203, 233). Even though an exact function of agnoprotein is still unknown, a role during virion assembly has been speculated (233). BKPyV agnoprotein has been found to be associated with lipid droplets (277), but the biological relevance of this is still unclear. Unlike BKPyV agnoprotein, JCPyV agnoprotein has been extensively studied. In the phosphorylated form, JCPyV agnoprotein primarily localizes to the cytoplasm, but in the dephosphorylated form, it translocates into the nucleus (212). JCPyV has been found to interact with LTag and thereby downregulate viral gene expression and DNA replication (240). JCPyV agnoprotein has also been suggested to function as a viroporin for the release of virions (269).

**microRNAs**

microRNAs (miRNAs) are small RNA molecules of approximately 22 nucleotides that regulate gene expression (21). BKPyV and JCPyV encode a pre-miRNA late in the infection that is processed into two functional 5p and 3p miRNAs homologous to SV40 miRNAs (256). BKPyV and JCPyV miRNAs are encoded by the early region but are transcribed by extension of the late transcript over the intergenic region into the early region. As a consequence, the miRNAs are perfectly complementary to a region in LTag mRNA. Hence, BKPyV and JCPyV miRNAs
downregulate LTag expression post transcriptionally in a small interfering RNA (siRNA)-like fashion by targeting and inducing cleavage of the early transcript (256, 271). Aside from their autoregulatory role, polyomavirus miRNAs have effects on host gene regulation. The 3p miRNAs of BKPyV and JCPyV which are identical, target and downregulate ULBP3, a cellular stress-induced ligand for NKG2D receptor of Natural Killer cells and thus play an immune evasive role (23). BKPyV encoded miRNAs have been implicated in viral latency or persistence (45).

**Viral replication**

The replication cycle of BKPyV has been characterized in primary human renal proximal tubular epithelial cells (RPTECs) and primary human urothelial cells (HUCs) (29, 178, 182). One replication cycle of BKPyV in RPTECs takes 48-72 hours and approximately 72 hours in HUCs. The replication cycle of JCPyV has not been studied in detail, however, early protein LTag and late protein VP1 expression at 24 and 48 hours postinfection (hpi), respectively in primary human fetal glial cells suggested that JCPyV also follows the pattern of the replication cycle of BKPyV (133, 218).
Figure 4. Schematic illustration of Polyomavirus replication. The numbers in the boxes indicate the order of the steps occurring during the replication. $^1$TruncTag is only expressed by BKPyV and $^2$T'ags are only expressed by JCPyV (modified from (58)).

The polyomavirus replication cycle (Figure 4) is described in detail below:

**Attachment:** The efficient attachment to the host cell receptor is a prerequisite for a productive viral infection. For the attachment, the binding pocket of the BC loops of VP1 interacts with the specific cell surface receptor (80, 104). BKPyV uses α (2,3)-linked sialic acid on N-linked glycoproteins or the gangliosides GD1b and GT1b as specific receptors (79, 183) while JCPyV uses terminal α (2,6)-linked sialic acid moiety present on lactoseries tetrasaccharide c (LSTc) as specific cellular receptors (164, 180, 209). In addition, the serotonergic receptor 5HT2AR has been suggested to be a specific receptor for JCPyV since JCPyV infection was rescued in non-
permissive HeLa cells (negative for 5HT2AR) by transfecting 5HT2AR (87). However, JCPyV infection was also found in 5HT2AR negative human brain microvascular endothelial cells, suggesting that it may serve as co-receptor or be dispensable for infection (51).

**Entry and uncoating:** Post attachment, BKPyV enters the host cells by caveolae mediated endocytosis while JCPyV enters the host cells by clathrin coated pit dependent endocytosis followed by caveolin-1-positive endosomes transport (201, 221, 229). Intracellular trafficking of BKPyV and JCPyV is not well understood. It is suggested that once internalized, BKPyV and JCPyV are transported to the endoplasmic reticulum via the cytoskeleton where partial or complete uncoating/denaturation occurs and this partially denatured capsid enters into the nucleus (14, 80, 183). When the closely related SV40 virion is released into the cytosol from the endoplasmic reticulum, the minor structural proteins VP2 and VP3 are exposed and promote the nuclear entry of SV40 DNA (207, 273). However, a study of JCPyV viral like particles (VLPs) showed that the nuclear localization signal of VP1 was involved in its nuclear entry through the nuclear pore complex (228).

**Early transcription and expression:** Immediately after nuclear entry, complete uncoating of the virion occurs followed by the early gene transcription by cellular RNA polymerase II and translation that produces the early proteins. LTag mediates cell progression to the S phase by interacting with the key tumor suppressor proteins pRB and p53. In addition, SV40 LTag was shown to induce the DNA damage response via ataxia telangiectasia mutated (ATM) protein or ataxia telangiectasia and Rad3-related (ATR) protein pathway, and this pathway was found to be essential for efficient viral replication (reviewed in (125)). JCPyV-infected cells expressing LTag have been found to accumulate in the G2 phase of the cell cycle as
a result of the activation of ATM and ATR-mediated G2 checkpoint pathways and this seems to facilitate JCPyV replication (215).

**Genome replication:** LTag is involved in the genome replication in at least two ways: first indirectly by stimulating the cell progression into the S phase, an important function since viral DNA replication is entirely dependent upon the cellular DNA synthetic machinery and second, LTag plays a direct role to initiate viral DNA replication. In more detail, LTag first undergoes oligomerization to generate a double hexamer and next, the Ori binding domain binds to the origin of replication of viral DNA via the consensus pentanucleotide G(A/G)GGC and unwinds the double stranded DNA in an ATP dependent pathway mediated by the ATPase domain (152, 190). Immediately after unwinding, LTag recruits the pol α/primase complex, polymerase δ and cellular replication proteins to the origin of replication and initiates bi-directional replication of the viral DNA (reviewed in(58)).

**Late transcription and translation:** At the onset of viral DNA replication, LTag activates the late promoter and initiates late gene transcription and translation that produces the three capsid proteins VP1, VP2 and VP3; and the non-structural agnoprotein. In addition, during the late phase of viral life cycle, LTag binds to the NCCR and reduces the activity of the early promoter thus autoregulating early transcription via a negative feedback mechanism (69).

**Assembly:** The specific sites of virion assembly are not well known yet (88). After the completion of the genome replication and the entry of the capsid proteins VP1, VP2 and VP3 to the nucleus, the packaging of DNA with cellular histones (minichromosome) is followed by polymerization of capsid proteins on the minichromosome leading to the icosahedral virions (99).
**Release:** The mechanism for progeny release is not well known. It is thought that over expression of viral proteins or the production of large amounts of progeny cause instability of the cell membrane leading to cell lysis. For SV40, a late protein denoted VP4 has been suggested to be involved in this process (64). There is also an open reading frame for a similar protein in the BKPyV and the JCPyV genomes.
BK and JC polyomavirus infection, diseases and treatment options

Primary infection, latency and reactivation

BKPyV and JCPyV which are transmitted independently of each other, infect a large proportion of the population without any specific signs or symptoms and then establish a latent infection in the renourinary tract (52). Interestingly, the primary infection of BKPyV commonly occurs during early childhood while that of JCPyV usually occurs during late childhood and adulthood (85, 162, 266). In a large study of 1501 blood donors and 721 children under 21 years of age, almost 80% of the 10 year old children and 87% of individuals between 21 to 50 years of age were seropositive for BKPyV but from that age, the seropositive was slowly declining (157). In the same study, JCPyV seropositive was found in about 20% of 10 year old children and was found to be continuously increasing with increasing age to reach the maximum of 51% by the age of 70 years. Although the exact percentages found in this study especially for JCPyV differ from the results found in other studies (85) possibly due to different methodology and population investigated, the trend is similar. Of note, in a study of HIV-1 infected patients, a JCPyV seropositivity of 90% was detected which suggested that risk factors associated with HIV-1 affect JCPyV transmission (132).

After the primary infection, sporadic reactivation causes asymptomatic urinary shedding of low levels BKPyV and/or JCPyV. In one study of 400 healthy blood donors from Switzerland, BKPyV or JCPyV DNA was found in the urine samples of 7 and 19% of the donors, respectively (85). However, in a smaller study of 20 female students where daily urine samples were examined over a period of two months, BKPyV DNA was found in urine samples from 55% of the students and JCPyV DNA from 10% of the students (161).
Even though the source of infection and mode of transmission is not completely resolved for either BKPyV or JCPyV, the fecal-oral route has been suggested based on the asymptomatic shedding of viruses in urine, the detection of the viral DNA in stool and saliva of healthy adults and in sewage (34, 85, 146, 281). Other suggested routes of transmission include transplacental transmission, transfusion of blood products, semen, and organ transplantation, particularly kidney allografts (reviewed in (135)).

In individuals with impaired immune function due to disease, pregnancy or treatment, reactivation of BKPyV in epithelial cells of the renourinary tract is more frequent and replication occurs at high level. This can lead to diseases, especially in KT and allogenic hematopoietic stem cell transplant (HSCT) patients (reviewed in (62)). Interestingly, shedding of JCPyV in urine is not connected to altered immune function but only a marker for ongoing replication in the urinary tract (132). However, JCPyV associated disease PML is almost exclusively affecting immunocompromised patients.

**Polyomavirus-associated nephropathy**

Although the first report describing a disease resembling polyomavirus-associated nephropathy (PyVAN) came in 1978, it was not before the late 1990s that PyVAN emerged, possibly due to the introduction of new and more effective immunosuppressive drugs such as mycophenolate mofetil and tracolimus (126). PyVAN is now recognized as the major viral complication in KT patients (129). Approximately 1-10% of all KT patients are affected by PyVAN within the first year after the transplantation (129). Although BKPyV is the major cause of PyVAN, JCPyV has been found to cause a few cases but this accounts for less than 1% of total PyVAN cases (75, 156).
The pathogenesis of PyVAN involves high-level BKPyV replication in epithelial cells lining the kidney tubules leading to necrosis, kidney dysfunction and subsequently allograft loss. During PyVAN, BKPyV with rearranged NCCRs emerge and they have an increased viral replication and give increase cytopathology compared to the archetype strain (111). Although immunosuppression is a major risk factor for PyVAN, the almost exclusively occurrence in KT patients suggests that factors specific to these patients are of importance (134). They include recipient determinants such as older age, male gender and negative BKPyV serostatus especially in pediatric recipients, donor determinants such as HLA mismatch, deceased donation, BKPyV load in the graft and female gender, graft determinants such as ureteric stents and modulating factors after transplantation like acute rejection or anti-rejection treatment.

The diagnosis of PyVAN is stratified into three categories: possible, presumptive and proven PyVAN based on quantitative PCR, cytology of urine and histopathological findings (134). In possible PyVAN, urine BKPyV DNA loads are high (>10⁷ Genome Equivalents (GEq)/ml), decoy cells are present but plasma is BKPyV negative. In presumptive PyVAN, plasma is BKPyV DNA positive (>10⁴ GEq/ml) in addition to high urine BKPyV DNA loads or decoy cells. In proven PyVAN, histopathological findings are present in addition to positive plasma and high urine BKPyV DNA loads (134). A proven PyVAN is further categorized into three different histopathological stages which indicate the severity of PyVAN. Stage A is characterized by mild viral cytopathic changes with minimal interstitial inflammation and fibrosis, and tubular atrophy and has a <10% risk of graft loss; Stage B is characterized by moderate to significant viral cytopathic changes and interstitial inflammation with moderate tubular atrophy and interstitial fibrosis and carries a 25-
75% risk of graft loss; and stage C is characterized with variable viral cytopathic changes and interstitial inflammation with extensive tubular atrophy and interstitial fibrosis and has >80% risk of graft loss (76, 134).

Of note, the focal nature of the infection can lead to false negativity in biopsy samples particularly in early stages of PyVAN (reviewed in (77)). To avoid this, a minimum of two core biopsies should be taken, preferentially containing medullary tissues (134). Urine cytology has been used as a screening test to detect epithelial cells bearing BKPyV inclusion, often called decoy cells. However, today, the most important tool for screening patients for PyVAN is quantitative PCR of urine and plasma. It is recommended to screen for BKPyV in urine or plasma every three months for the first two years after transplantation. More than $10^7$ and $10^4$ GEq/ml of BKPyV DNA in urine and plasma, respectively, indicate the development of PyVAN (131). The absence of viruria practically rules out PyVAN (reviewed in (77)). Early diagnosis and early treatment of PyVAN has increased the graft survival (128).

**Polyomavirus-associated hemorrhagic cystitis**

Hemorrhagic cystitis is defined as a hemorrhagic inflammation of the urinary bladder mucosa leading to painful hematuria, severe bladder hemorrhage, and possibly to clot-related urinary retention and kidney failure. Hemorrhagic cystitis is categorized into an early onset hemorrhagic cystitis that occurs in HSCT patients within days following chemotherapy and irradiation; and a late onset hemorrhagic cystitis which is also called infectious hemorrhagic cystitis, occurring about 50 days after HSCT. The late hemorrhagic cystitis is associated with reactivation of urotropic viruses such as BKPyV, adenovirus and cytomegalovirus (173). A hallmark of polyomavirus-associated hemorrhagic cystitis (PyVHC) is high-level BKPyV replication in the urothelial cells lining the bladder. Although the pathogenesis of
PyVHC is not fully understood, the following sequence of events has been suggested (20, 31). First, urotoxicity of the conditioning protocol causes subclinically damage to the urothelium. Next, during the aplastic phase, immunologically uncontrolled BKPyV replication is occurring leading to urothelial denudation, urine leakage, and hematuria. Finally, upon engraftment a stronger inflammation and cytopathic damage is seen (reviewed in (130)). The incidence of PyVHC after allogeneic HSCT is 5-15% (130). The risk factors associated with PyVHC are allogeneic versus autologous HSCT, myeloablative conditioning, a transplant from an unrelated donor, cord blood transplantation and BKPyV seropositive prior to transplantation (reviewed by (108)).

The diagnosis of PyVHC requires the triad of cystitis, hematuria and high-level BKPyV replication with urine BKPyV DNA load of >10^7 GEq/ml (130). In a study on children, BKPyV viremia preceded PyVHC (168) and in a study on adults viremia was correlated with the severity of the disease (108).

**Progressive multifocal leukoencephalopathy**

PML is usually a fatal demyelinating disease of the central nervous system (CNS) in immunosuppressed individuals caused by the cytopathic replication of JCPyV in myelin-producing oligodendrocytes. PML was first described in 1958 in a patient with chronic lymphocytic leukemia and Hodgkin’s lymphoma (16). It was a rare complication of immunocompromised particularly neoplastic patients until the pandemic of HIV started. During the 1980s, about 5% of HIV positive patients developed PML and this patient group still constitutes approximately 80% of all PML cases (27). Recently, an increased incidence of PML was reported in patients with autoimmune diseases who underwent immunomodulatory therapies. The incidence rate of PML was up to 11.1 cases per 1000 multiple sclerosis patients treated with natalizumab, a monoclonal antibody that prevents T and B lymphocytes migration
into the brain, if the patient was JCPyV seropositive, had taken immunosuppressants before the initiation of natalizumab and had received 25 to 48 months of natalizumab treatment (33). An incidence of 1 in 500 was seen in psoriasis patients treated with efalizumab, a monoclonal antibody that prevents T and B lymphocytes migration from blood vessels into the tissues (91). PML also occurs in 1 of 2500 rheumatoid arthritis patients treated with rituximab, a monoclonal antibody that depletes B cells from the peripheral circulation and cerebrospinal fluid (CSF) (50, 55). Similarly, though less frequently, PML has been reported in patients with impaired immune functions for other reasons such as solid organ transplant (SOT) and allogenic HSCT, as well as idiopathic CD4 lymphocytopenia (10, 27, 68, 133) and sometimes in individuals with minimal or occult immunosuppression (106, 113, 205).

Recently, three different but not mutually exclusive hypothesis were postulated for PML pathogenesis (132). i) After primary infection, JCPyV reaches and remains latent in the CNS. When the JCPyV specific T cell level decreases in the CNS, JCPyV reactivates and causes cytolytic replication in the oligodendrocytes with ensuing progression to PML. ii) After primary infection, JCPyV persists in other cells of the body, i.e. lymphocytes or hematopoietic progenitor cells and reactivates when JCPyV specific T cell level decreases. Then, via occult viremia, JCPyV reaches the CNS causing cytolytic replication leading to PML. iii) After primary infection, JCPyV persists in lymphocytes or bone marrow progenitor cells and these latently infected cells migrate to the CNS where the virus reactivates causing cytolytic replication under the condition of decrease or absence of JCPyV specific T cells.

The diagnosis of PML is very challenging and requires recognition of the clinical presentation, backed up by compatible findings in magnetic resonance imaging of the brain. The clinical presentations are variable but generally include progressive focal
neurological deficits of mostly motor, cognitive and visual functions (132). Demonstration of JCPyV DNA in CSF is confirmatory but frequently negative in the early stages of disease. Not uncommonly, repeated CSF testing and/or brain biopsy is necessary. As such, the diagnosis demands a high degree of clinical suspicion and perseverance. Detection of JCPyV DNA in the plasma is not helpful as many PML patients are not viremic (91) and plasma JCPyV DNA has not been accepted as a marker of PML disease (10).

Since diagnosing PML can be difficult, the three different terms are used: i) Possible PML which defines those patients who are at high risk with the relevant clinical and radiological signs but where JCPyV DNA is undetectable in CSF by PCR. ii) Probable PML defines patients with PCR positive JCPyV DNA in CSF and have multifocal neurological deficits and corresponding radiological findings. This is also called virologically or laboratory confirmed PML. iii) In proven PML, also referred to as histologically proven PML, a brain biopsy of the affected area shows demyelination with viral cytopathic alterations surrounded by macrophages and atypical astrocytes. JCPyV involvement is demonstrated by either positive immunohistochemistry for LTag or VP1 positive in situ hybridization (reviewed in (132)).

Other diseases associated with BKPyV and JCPyV

BKPyV-associated ureteric stenosis is reported in 2-6 % of KT patients (reviewed by (280)). In fact, the first BKPyV isolation was made in a KT patient suffering from ureteric stenosis (100). The pathogenesis of ureteric stenosis is still not completely resolved. It is suggested that high doses of steroids in the KT patients may permit high-level BKPyV replication in the urothelium and underlying smooth muscle cells further contributing to pre-existing ureteric injury (59, 204).
Apparently, ureteric stenosis is now less frequently reported possibly due to a decline in the use of ureteral stents (42, 129). Of note, irreversible BKPyV-associated ureteric stenosis has not been reported in non-KT patients (280). The treatment options include percutaneous nephrostomy and concurrent reduction of immunosuppression (280).

**BKPyV-associated diseases of the CNS** have been reported. BKPyV was found to be associated with meningoencephalitis in two HIV positive patients and an SOT patient; and with PML-like disease in a KT patient (41, 114, 136, 278). Recently, a case of BKPyV-associated PML was reported in an immunocompromised patient (65).

**The potential role of BKPyV and JCPyV in cancer** has been demonstrated by the capability of their early T antigen proteins to induce transformation of cells *in vitro* and cause tumors in laboratory animals (reviewed in (284)). BKPyV or JCPyV DNA/proteins have been found in different human neoplasms (reviewed in (62, 132, 234, 284)). In 2012, WHO international agency for cancer Research Monograph Working Group classified BKPyV and JCPyV as possibly carcinogenic to humans (group 2B) based on the inadequate evidence in humans and sufficient evidence in experimental animals (39).

**JCPyV-associated granule cell neuronopathy** was described in HIV positive patients, in a natalizumab treated multiple sclerosis patient and in a rituximab treated non-Hodgkin lymphoma patient (63, 78, 165, 246). JCPyV-associated granule cell neuronopathy is caused by the cytopathic replication of JCPyV in granule cell neurons of the cerebellum.
**JCPyV-associated encephalopathy** is caused by the cytopathic replication of JCPyV in cortical pyramidal neurons and was reported in a HIV negative lung cancer patient (289).

**JCPyV-associated meningitis** was first described when JCPyV DNA was detected by PCR in the CSF of two immunocompetent patients with suspected meningitis (25). Recently, a definitive case of JCPyV-associated meningitis was reported in an immunocompetent individual (5). The CSF was JCPyV DNA positive and immunohistochemistry staining of brain tissue showed significant JCPyV infection of the leptomeningeal cells of the pia mater surrounding the cerebrum and cerebellum.

**Treatment of polyomavirus-associated diseases**

There is still no effective anti-BKPyV or anti-JCPyV drug available. One of the reasons for this is that these small viruses encode only few proteins and therefore have only few targets for direct acting agents.

For KT patients with PyVAN, the best available treatment option is to regain immune function by cautiously reducing or changing the immunosuppressive drugs. If this is performed at an early stage of the disease, this may control the viral replication, clear BKPyV viremia and stop the progression of PyVAN (109, 119, 245). Unfortunately, this treatment option at the same time increases the risk of acute rejection (151). Even more, the restoration of immune function is not always possible or enough to control the viral replication, therefore, drugs like cidofovir, leflunomide, and fluoroquinolones and intravenous immunoglobulin have been used as adjunctive therapy especially in a combination with the reduction of immunosuppression (reviewed in (151)). Cidofovir is a nucleoside analogue that is licensed to treat resistant human cytomegalovirus (HCMV) infection in HIV positive patients (234).
Cidofovir effectively reduced BKPyV replication in primary RPTECs, but gave considerable cytostatic effects (29) and is also known to be nephrotoxic and can give irreversible acute kidney failure (137, 216). Leflunomide is an inhibitor of de novo pyrimidine synthesis and is licensed for the treatment of rheumatoid arthritis (234). Leflunomide also inhibits BKPyV replication in primary RPTECs but with significant cytostatic effects (30) and has been reported to give anemia, liver damage, lung toxicity and thrombotic microangiopathy (19, 170, 237). Although some case reports showed favorable outcome of PyVAN after treatment with either cidofovir or leflunomide, a systemic review of the studies published until 2009 showed that there was no clear benefit of these drugs over the reduction of immunosuppression alone (151). The fluoroquinolones are synthetic broad spectrum antibacterial agents which inhibit the two bacterial enzymes topoisomerase II and IV (159). It has been speculated that fluoroquinolones can inhibit the helicase activity of BKPyV LTag as described for SV40 LTag (8, 259). The fluoroquinolones ofloxacin and levofloxacin were found to inhibit BKPyV replication in primary RPTECS (259). The mechanism for this could be by the inhibition of LTag helicase activity, but since the replication of cellular DNA was reduced, the inhibition of cellular topoisomerase II was also suggested. Fluoroquinolones have been investigated in some non-randomized studies as prophylaxis or treatment of BKPyV infection after KT or HSCT with favorable and unfavorable outcomes (97, 167, 172, 276, 286). At the moment, there is one ongoing phase 4 study where ciprofloxacin is given as prophylactic the first 6 months after the KT (Clinical Trials.gov NCT01789203).

Intravenous immunoglobulin contains pooled IgG from the plasma of approximately 1000 blood donors (226, 234) and contain neutralizing antibodies against BKPyV that can inhibit in vitro BKPyV infection (230). Intravenous
immunoglobulin has been used as treatment of BKPyV infection with variable success (255, 283). But, in two patients, a significant increase of urine and plasma BKPyV load was seen after intravenous immunoglobulin administration and the patients progressed to PyVAN (38, 186). The fact that some KT patients with high titers of BKPyV IgG developed PyVAN raises a question about the benefit of this treatment (35).

While PyVAN partly results from reduced immune surveillance, PyVHC is partly caused by recovery of the cellular immune response upon engraftment (62). The treatment of PyVHC is, therefore, mainly supportive involving symptomatic analgesia, hyperhydration, diuresis and continuous bladder irrigation to prevent clot formation and urinary tract obstruction; and substitution of platelets and erythrocytes (130). As for PyVAN, adjunctive prophylaxis or treatment with cidofovir or fluoroquinolones has been used without any documented benefit (234). Moreover, hyperbaric oxygen either alone or in combination with intravesicular cidofovir has been successfully used to treat PyVHC (95, 120, 243). The hyperbaric oxygen is believed to stimulate the repair of mucosa in the urinary bladder (95).

So far the only efficient treatment of PML is to regain immune function. In HIV/AIDS patients, this can be achieved by the start of HAART/cART treatment, in SOT patients by the reduction of the immunosuppressive therapy and in patients with autoimmune disease by removing the immunomodulatory therapies. However, the rapid increase in immune function leads to immune-reconstitution inflammatory syndrome in 40% of all PML patients with mortality rate up to 53% (115). Many broad spectrum nucleoside analogues such as cytosine arabinoside, adenosine arabinoside, azidothymidine, acyclovir and cidofovir have been used to inhibit JCPyV replication in PML patients without much success (91, 189). Additionally, mefloquine,
an antimalarial drug, was found to inhibit JCPyV replication in vitro (43) and has been used successfully to treat some PML patients (3, 26, 160, 194, 206, 261, 291) while it was unsuccessful for others (56, 113, 163, 200, 241). In a clinical trial comparing CSF JCPyV DNA load in PML patients receiving standard care with those who received standard care plus mefloquine, no significant difference was found (56).
Artesunate

Introduction

Artemisinin, and its derivatives artesunate (Figure 5), artether, artemether and the active metabolite dihydroartemisinin (Figure 5) are well known antimalarial drugs with high tolerability and a good safety record (61). In 2005, World Health Organization officially recommended artemisinin and its derivatives for the treatment of malaria particularly in a combination with other antimalarial drugs to avoid resistance development. Additionally, artesunate was found to have antiviral, antiparasitic, antifungal, anti-inflammatory and anti-allergic properties both in vitro and in vivo (reviewed in (138)).

![Figure 5](image.png)

**Figure 5.** Schematic illustration of chemical structure of artesunate and its active metabolite dihydroartemisinin showing endoperoxide bridge inside the ring.

Tu and his colleagues at the China Academy of Chinese Medical Sciences were searching for an active compound against malaria and in 1972, they extracted artemisinin from the Chinese medicinal plant *Artemisia annua* L. and found this to be very efficient against parasitemia in mice and monkeys infected with malaria
parasites (274). Artemisinin is a sesquiterpene lactone consisting of a distinctive endoperoxide bridge which is considered to be responsible for its antimalarial activity. Since artemisinin was poorly soluble in water or oil and could not be administered to the patients intravenously, several derivatives from artemisinin have been developed such as the oil soluble artemether and artether as well as the water soluble artesunate. In fact, these derivatives were developed from dihydroartemisinin which was produced by reducing the lactone group of artemisinin to a lactole (hemiacetal function) by sodium borohydride (reviewed in (61, 171)). Of note, dihydroartemisinin was the first derivative made, but it was as poorly soluble in water and oil as was its parental drug (12, 174) and later it was recognized as the active metabolite of artesunate and other artemisinin derivatives.

Artesunate is a succinate esterified dihydroartemisinin (171) and it is the only derivative that can be given to patients orally, intravenously, intramuscularly and rectally. It has remarkable activity against *Plasmodium falciparum* and *Plasmodium vivax* and is highly effective in the treatment of severe malaria (83). *In vivo*, artesunate is rapidly and extensively converted through esterase-catalyzed hydrolysis to dihydroartemisinin and artesunate is therefore considered a prodrug. Interestingly, during *in vitro* testing against *Plasmodium falciparum*, both artesunate and dihydroartemisinin was found to be equally potent and significantly more active (3-5 fold) and also toxic than the other artemisinin derivatives (175, 177). During elimination, dihydroartemisinin is resolublized and inactivated by glucuronidation in the liver followed by excretion in the urine (102, 143).

A meta-analysis of 16 randomised malaria treatment trials conducted for artesunate in combination with other drugs (chloroquine or amodiaquine or sulfadoxine-pyrimethamine or mefloquine) did not find any significant adverse effects
Introduction

of artesunate (4). Because of its high safety profile and tolerability, the potency of artesunate in other areas has also been extensively studied. As a result, artesunate and dihydroartemisinin were found to inhibit the proliferation of a wide variety of cancer cells in vitro and to reduce tumor size in animal models (review in (138)). Artesunate has been used to treat cancer patients and some case studies showed that artesunate reduced the tumor growth of laryngeal squamous cell carcinoma and metastatic uveal melanoma and increased the survival rates of the patients (28, 263). In a randomized control trial of 120 advanced non-small cell lung cancer, artesunate combined with a chemotherapy regimen of vinorelbine and cisplatin was found to increase the short term survival and prolong the cancer progression time (the full article is in Chinese) (293).

Mechanism for antimalarial and anticancer activity

The mechanism for the antimalarial and anticancer activity of artesunate and other artemisinins is not understood yet (71, 74). However, it is believed that the endoperoxide bridge is responsible for both activities (reviewed in (71, 93, 138)). The endoperoxide bridge undergoes hydrolysis and produces toxic radicals such as, for example, reactive oxygen species (ROS) or a carbon centered radical, a highly potent alkylating agent. Interestingly, the hydrolysis of the endoperoxide bridge is greatly enhanced by the presence of free iron (82). The suggested antimalarial mechanism is that these toxic radicals interfere with the heme detoxification pathway, inducing alkylation of the \textit{plasmodium falciparum} translationally controlled tumor protein (PfTCTP) encoded by the \textit{pftctp} gene and other proteins. This may inhibit the sarco-endoplasmic reticulum calcium dependent ATPase (SERCA) protein encoded by the \textit{PfATPase6} gene, or interfere with \textit{plasmodium} mitochondrial functions (reviewed in (71)). Similarly, for anticancer activity, the toxic radicals induce direct
oxidative cellular damage and as a result, a wide variety of effects have been found, including cell cycle arrest, induction of apoptosis, alteration of hormone responsive properties and/or inhibition of angiogenesis in a variety of human cancer cells (reviewed in (93, 138)). Since most of the cancer cells are enriched with iron compared to normal cells (reviewed in (153)), they are more prone to be affected.

**Antiviral activities**

The first hint of antiviral properties of artemisinin against influenza A virus was already provided by Chinese Scientists in the 1980s (227). In 2002, Efferth and his colleagues observed antiviral properties of artesunate while investigating its antiproliferative activities in cell culture (83). Artesunate was found to inhibit the replication of a wide variety of human viruses (**Figure 6** and **Table 2**). The extensive studies on HCMV in vitro demonstrated that artesunate is equally effective against both ganciclovir sensitive and resistant strains (54, 83). Since activation of HCMV replication is differently regulated in different types of cells, the efficacy of artesunate against HCMV was tested in a wide variety of human cells including astrocytoma, foreskin- and lung-fibroblast cells and the results showed a strong inhibitory effect in all cells tested (54, 83, 94, 155, 195, 239). However, there was some variation in the half maximal effective concentration (EC$_{50}$) (**Table 2**) and it was therefore suggested that artesunate had some cell type specificity (54).
Dihydroartemisinin has also been investigated for its anti-cytomegalovirus activities in vitro but the studies showed that it was less effective compared to artesunate (54, 94). However, the results were not straightforward. At low dose of about 1.6 microMolar (µM), dihydroartemisinin and artesunate inhibited HCMV replication to the same extent, but at higher doses, dihydroartemisinin showed less effect than at a lower dose and was significantly less effective than artesunate (54). In another in vitro study of HCMV replication, artesunate was found to be superior to dihydroartemisinin when given as a single dose but, after fractional doses, dihydroartemisinin was equal to or even more effective than artesunate (94). This could perhaps be explained by the poor stability of dihydroartemisinin compared to artesunate (121).
Table 2. The half maximal effective concentration (EC$_{50}$) of artesunate for different viruses in different cells. With this concentration, the viral replication is inhibited by 50%.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Artesunate EC$_{50}$ (µM)</th>
<th>Host cell</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCMV AD169- Laboratory strain</td>
<td>5.8±0.4</td>
<td>HFF</td>
<td>(83)</td>
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<td>6.9±0.2</td>
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<td>(83)</td>
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<td>(155)</td>
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<td>MRC-5</td>
<td>(247)</td>
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<td>(247)</td>
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<td>HCMV Ganciclovir/ Foscarnet/Cidofovir resistant</td>
<td>4.62±1.91</td>
<td>HEL</td>
<td>(54)</td>
</tr>
<tr>
<td>HCMV Ganciclovir/ Foscarnet/Cidofovir resistant</td>
<td>2.62±0.75</td>
<td>HFF</td>
<td>(54)</td>
</tr>
<tr>
<td>HCMV clinical isolate</td>
<td>2.16±1.08</td>
<td>MRC-5</td>
<td>(94)</td>
</tr>
<tr>
<td>HHV-6A U1102</td>
<td>3.80±1.06</td>
<td>HSB-2</td>
<td>(195)</td>
</tr>
<tr>
<td>HBV (HBsAg and HBV DNA)</td>
<td>2.3 &amp; 0.5</td>
<td>HepG2 2.2.15</td>
<td>(235)</td>
</tr>
<tr>
<td>BK polyomavirus</td>
<td>4.2</td>
<td>RPTECs</td>
<td>(260)</td>
</tr>
</tbody>
</table>

MRC-5: Human lung fibroblasts
HFF: Human foreskin fibroblasts
Raji: Human B lymphoblast
U373MG: Human astrocytes
HEL: Human embryonic lung
HSB-2: Human T lymphoblast
RPTECs: Human primary renal proximal tubular epithelial cells
HepG2 2.2.15: Human hepatoma cells transfected with HBV DNA
293T: Human embryonic kidney cells transformed with SV40 LTag
Introduction

As with the antimalarial and anticancer activities of artesunate, the molecular mechanism for the antiviral effect of artesunate is not yet completely understood. Artesunate inhibits the expression of HCMV immediate early genes and the viral DNA replication (17, 83). This inhibition is suggested to be connected to the interference of artesunate with the central regulatory pathways of HCMV infected cells. By inhibiting NF-kB or Sp1 dependent activation pathways, artesunate interferes with critical metabolic requirements for HCMV replication (84). Again the importance of endoperoxide in the inhibition of HCMV by artesunate is further underscored by the lack of inhibition of HCMV by an artemisinin deoxy dimer lacking endoperoxide (123). Of note, as found for the antimalarial and anticancer activities, iron also increased the activity of artesunate against HCMV in vitro (155).

Due to its strong antiviral activity against a variety of herpes virus in cell culture, artesunate has been used to treat herpes virus diseases. To our knowledge, a total of 14 patients, 9 HSCT, 4 SOT and 1 pediatric patient with heart disease, have been treated with oral or intravenous artesunate for viral infections and of these, 7 patients cleared with the viral infection (105, 116, 169, 254, 258, 287). Two HSCT and two SOT (1 KT and 1 lung transplant) patients with multidrug resistant HCMV infection were orally treated with artesunate at a dose of 100-200 mg/day; and favorable clinical and virological response was reported at 10-30 days for HSCT and 1-3 months for SOT patients after the treatment started (105). The treatment was continued for 1 to 7 months. Of note, one of the HSCT patients received foscarnet in combination with artesunate. In addition, one HSCT patient with multidrug resistant herpes simplex virus-2 (HSV-2) infection was successfully treated with oral artesunate 100 mg per day for 30 days (254). This patient had two more episodes of the same viral infection, but also this was successfully treated with artesunate. Also a
patient with severe heart failure was successfully treated for human herpes virus-6B (HHV-6B) infection (116). In this patient, artesunate was given intravenously at a dose of 5 mg/kg/day for 10 days and then continued orally with 2X5 mg/kg/day for 10 more days. On other hand, artesunate failed to cure valganciclovir resistant HCMV infection of KT patients after a 30 day treatment (169). In a clinical trial, pre-emptive treatment for HCMV infection in 5 HSCT patients was tested. Oral artesunate at a dose of 2X200 mg per day for one day, followed by 100 mg/day for 28 days was given, but this was successful in one of the 5 patients (www.clinicaltrials.gov NTC00284687) (287). For some of the unsuccessfully treated patients, a reduction of the viral load was seen (105), which might indicate some effect. To summarize the results of these studies, by curing 7 of 14 patients, artesunate had a success rate of 50%. As part of two randomized malaria treatment trials, artemisinin at a dose of 4 mg/kg/day was given to children for 3 days, and this did not reduce HCMV infection when investigated by qPCR of dried blood samples collected (98). However, in this study, the artemisinin treatment was given for a short period.
Aims of the study

The aim of this thesis was to explore the possible future use of the antimalarial drug artesunate as a treatment for PyVAN, PyVHC and PML by investigating the antiviral effect on BKPyV and JCPyV replication in authentic cell culture models.

Paper I

PyVAN is caused by high-level lytic replication of BKPyV in kidney tubular epithelial cells in the graft of KT patients. The aim of this study was to perform a detailed investigation of the effects of artesunate on BKPyV replication in primary human RPTECs, the target cells for BKPyV in PyVAN.

Paper II

High-level replication of BKPyV in urothelial cells is a common feature of PyVAN and PyVHC. The aim of this study was to characterize the replication of BKPyV in primary HUCs from bladder and compare it to BKPyV replication in RPTECs.

Paper III

PyVHC pathogenesis involves high-level replication of BKPyV in bladder urothelial cells. The aim of this study was to investigate the effect of artesunate on BKPyV replication in primary HUCs, previously characterized in paper II.

Paper IV

PML is caused by high-level lytic replication of JCPyV in oligodendrocytes. Since primary human oligodendrocytes are difficult to obtain and propagate, the aim of this study was to first compare the permissivity of COS-7, HEK 293TT, SVG-A and M03.13 cell lines for JCPyV replication; and second to investigate the effect of artesunate on JCPyV replication in the most permissive cell line tested.
Summary of the papers

Paper I

Artesunate, a widely used antimalarial drug, has shown antiviral activity against a wide variety of herpes viruses (Table 2). Here, we investigated the effect of artesunate on BKPyV replication in RPTECs, the major target cells in PyVAN. Artesunate inhibited BKPyV replication in a concentration-dependent manner with a 50% effective concentration (EC₅₀) of 4.2 µM. Artesunate at 10 µM reduced the extracellular BKV load by 65% at 72 hpi. The inhibition was seen on early LT ag and late VP1 mRNA and protein expression; and on BKPyV DNA replication. Importantly, the proliferation of RPTECs was also inhibited in a concentration-dependent manner. At 72 hpi, artesunate at 10 µM reduced cellular DNA replication by 68% and total metabolic activity by 47%; these reductions were connected to cytostatic but not a cytotoxic effect and the cells were arrested in G₀ or G₂ phase of cell cycle. Both the antiproliferative and antiviral effects of artesunate at 10 µM were reversible. In conclusion, artesunate inhibits BKPyV replication in RPTECs by inhibiting BKPyV gene expression and genome replication. The antiviral mechanism appears to be closely connected to cytostatic effects on host cells, underscoring the dependence of BKPyV on host cell proliferative functions.

Paper II

High-level BKPyV replication in HUCs is a hallmark of PyVHC and PyVAN. We characterized BKPyV replication in HUCs and compared it to the replication in RPTECs. HUCs were easily infected, as shown by expression of LTag, VP1-3, and agnoprotein, and intranuclear virion production. Compared to RPTECs, progeny release was delayed by ≥ 24 hours. BKPyV-infected HUCs rounded up like “decoy cells” and detached without necrosis. In conclusion, BKPyV infection of HUCs and
RPTECs is significantly different and the data support the notion that PyVHC pathogenesis is not solely due to BKPyV replication, but likely requires urotoxic and immunological cofactors.

**Paper III**

Here, we investigated the effect of artesunate on BKPyV replication in HUCs, the major target cells in PyVHC but is also targeted in PyVAN. Artesunate inhibited BKPyV replication in HUCs with an EC\textsubscript{50} of 6.2 µM. No inhibition was seen on BKPyV DNA replication. Artesunate inhibited cell proliferation and concentrations from 10 µM resulted in a slightly reduced number of viable cells at 72 hpi. Of note, a single dose of 20 µM produced stronger effect than multiple doses of 10 µM. In conclusion, artesunate affects late steps in the BKPyV replication cycle in HUCs and this is closely linked to significant cytostatic effects.

**Paper IV**

High-level lytic replication of JCPyV in oligodendrocytes causes PML, an often fatal disease of brain, mainly affecting immunosuppressed individuals. Here, we investigated the effect of artesunate on JCPyV replication in COS-7 cells. The permissivity for JCPyV MAD-4 was first compared for HEK 293TT, SVG-A and M03.13 cell lines; and COS-7 was selected for the study. Artesunate caused a concentration-dependent decrease in extracellular JCPyV DNA load with an EC\textsubscript{50} of 3.0 µM at 96 hpi. This effect correlated with an inhibition of the expression of VP1, production and release of infectious viral progeny. For concentrations below 20 µM, a transient reduction in DNA replication and cell proliferation was seen, while for higher concentrations some cytotoxicity was detected. Interestingly, the JCPyV-infected cells were more sensitive to the cytostatic effect of the drug than the mock-infected cells. A selective index of 15 was found when cytotoxicity was calculated based on
cellular DNA replication. In conclusion, artesunate inhibits JCPyV replication in COS-7 cells at µM concentrations and the inhibition probably reflects an effect on cellular proteins and involves transient cytostatic effects.
General discussion

High-level BKPyV replication in kidney tubular epithelial cells and bladder urothelial cells causes PyVAN in KT patients and PyVHC in allogeneic HSCT patients, while lytic replication of JCPyV in oligodendrocytes in the context of immunosuppression causes PML. These serious diseases remain significant challenges since no effective and safe antiviral treatment is available (132, 234). Currently, the restoration of virus-specific immune functions in patients with PyVAN or HIV positive PML patients is the only way to control and reverse these replicative diseases. However, the recovery of immune functions has significant clinical limitations, because of undesired side effects such as transplant rejection and/or immune-reconstitution inflammatory syndrome in patients with PyVAN and PML, respectively. Moreover, this strategy may not be rapidly available in some transplant patients and almost impossible to achieve in a timely fashion in cancer patients. In PyVHC patients, the inflammatory response following the recovery of the cellular immune response may actually be part of the pathogenesis, although further studies are needed to identify the key mechanisms (62). Thus, antiviral therapies are expected to play a significant role in all patients affected by BKPyV and JCPyV diseases to either extend the time window for immune reconstitution or to primarily block viral replicative damage. In some studies, certain drugs have been used with only limited or no evidence of antiviral efficacy for BKPyV and JCPyV replication, but sometimes severe side effects instead (19, 137, 170, 216, 237). Therefore, the search for safe drugs with antiviral properties against BKPyV and JCPyV is clearly warranted today. Given the long process of drug development and licensing, it is of interest to identify drugs that are already in clinical use for other indications, but may have
convincing antiviral properties. This could dramatically shorten the time to clinical use and even provide some experience in compassionate or off-label use.

The aim of this PhD project was to investigate the effect of the antimalarial drug artemether on BKPyV and JCPyV replication in cell culture. Artesunate caught our attention since this drug has been safely used by millions of people with severe malaria (73), the drug has shown broad antiviral properties in vitro (Table 2) (17, 83, 84, 195, 235, 236) and has been reported to be successfully used in patients with multiresistant HCMV (105, 258), HSV-2 (254) and HHV-6B infection (116). In our work, we show for the first time that artemether inhibits BKPyV replication in RPTECs and in HUCs, in a concentration-dependent manner (Paper I and III). The inhibition of viral replication in vitro is closely connected to inhibition of host cell proliferation. However, for RPTECs, this inhibitory effect was only transient. Although the precise mechanism of BKPyV inhibition is still undefined, we have some indications that the difference of the host cell may play a different role, since the viral replication cycles in RPTECs and HUCs were affected at different steps. We also observed that artemether inhibits the replication of JCPyV in COS-7 cells (Paper IV). Also here the inhibition is closely connected to a transient inhibition of cell proliferation. Taken together, the work shows promising effects of artemether on BKPyV and JCPyV replication in all cells tested, but also some cell type-dependent cytostatic effects. The inhibitory effects were seen at concentrations of 1.25 to 10 µM. At very high concentration of ≥ 20 µM, some cytotoxic effects were seen. The significance and the potential application of this work will now be discussed in more detail.
Relevance of the cell culture model

Before any potential antiviral drug is tested on humans, evaluation of the safety and efficacy of the drug should be tested in appropriate cell culture and if possible in animal models. Since there is no known animal model for BKPyV or JCPyV replicative diseases, the choice of cell culture model is particularly important. For the study of the antiviral activity of artesunate on BKPyV replication, we used RPTECs (Paper I) and HUCs (Paper III). Both, RPTECs and HUCs represent the target cells of BKPyV in pathologies of PyVAN and PyVHC. For the study of JCPyV antiviral, COS-7 cells were used, which have been widely used by other researchers because of their permissiveness to JCPyV replication (Paper IV). One very important question is if these were well justified cell culture model systems.

For PyVAN, an important part of the pathogenesis is the undisturbed high-level BKPyV replication in the kidney tubular epithelial cells. When kidney biopsies from patients with PyVAN were examined, BKPyV intranuclear inclusion bodies in epithelial cells along the entire nephron i.e. the glomerulus, proximal- and distal convoluted tubules, collecting tubules and to a lesser extent the loop of Henle, were observed (192, 210). Interestingly, immunohistochemistry also revealed that urothelial cells in these patients were infected by BKPyV, although this was clinically silent (210). BKPyV was mainly found in superficial transitional cells but occasionally also in basal cells. Of note, the infected urothelial cells in PyVAN patients have been suggested to account for more than 90% of the urine BKPyV DNA load and can probably influence the disease progression due to reflux of virus containing urine into the graft (96). Based on this, RPTECs and HUCs seem to be the best model systems to investigate the effect of new drugs for a potential future treatment of PyVAN. Commercially available RPTECs and HUCs were therefore used for these studies. Since RPTECs
up to the 6th passage have been found to provide a similar environment as the cells in vivo (reviewed in (46), all experiments with RPTECs were performed with cells at passage 4. Apparently, RPTECs remain in a differentiated state with expression of organic anion transporters and members of the ATP binding cassette transporter family (2, 46, 140). Moreover, BKPyV replication in RPTECs was already characterized and RPTECs had been used for several previous antiviral studies (29, 30, 182, 232, 259). Studies on HUCs have shown that these cells change in culture from the transitional epithelial phenotype to a proliferative basal/intermediate cell phenotype (141, 181, 264). In line with this, real-time viability monitoring using the xCelligence system demonstrated that HUCs proliferated in vitro (Paper II and III), although to a lesser extent than RPTECs (Paper I) and COS-7 cells (Paper IV). Except for two papers where mixed cells from the urine of newborn infants were isolated and infected with BKPyV (24, 89, 238), there was no prior published work on BKPyV-infection of primary urothelial cells and we therefore made an effort to characterize this in detail (Paper II). To avoid unnecessary cell differentiation and to endeavor similar conditions, all experiments with HUCs were performed with cells at passage 4.

For PyVHC, one important factor in the pathogenesis is an uncontrolled high-level BKPyV replication in urothelial cells. Therefore, primary human urothelial cells like HUCs seem to be the best model system for investigation of new antiviral drugs for treatment of PyVHC. In fact, in wells with BKPyV-infected HUCs, we observed cells rounding up to form decoy-like cells and denuded areas (Paper II), nicely mimicking the in vivo situation in an infected bladder.

PML is caused by the cytopathic replication of JCPyV in the myelin-producing oligodendrocytes (133). Immunohistochemistry of brain biopsies from PML patients
often reveal astrocytes staining positive for LTag and sometimes VP1 (10), but these cells are probably not productively infected but have taken up virus or cellular and viral debris (133). Except for human primary fetal glial cell (218, 219), and brain progenitor-derived astrocytes (112), hardly any primary human cells are permissive for JCPyV (133). Although human primary oligodendrocytes would have been the best cell model for our study of artesunate treatment of JCPyV replication, oligodendrocytes are difficult to obtain and difficult to propagate unless they are immortalized. Besides this, ethical issues, poor availability and cumbersome preparation prevented us from preparing primary oligodendrocytes. To try to overcome these obstacles, we ordered the immortalized human-human hybrid cell line M03.13 with phenotypic characteristics of primary oligodendrocytes (191), but unfortunately we were not able to infect these cells with JCPyV MAD-4 (Paper IV). We therefore decided to use the human fetal glial cell line SVG p12 (ATCC CRL-8621) derived from human fetal glial cells immortalized with a plasmid conferring constitutive SV40 LTag expression (187). Surprisingly, we found that these cells contained infectious BKPyV (Appendix II) (127) and they could therefore not be used. We kindly requested and received SVG-A cells, a subclone of SVG cells, from Professor Walter Atwood at Brown University USA, and found that they were BKPyV negative and therefore could be used (Appendix II). Nevertheless, before we started our antiviral study, we decided to compare JCPyV replication in SVG-A, HEK 293TT and COS-7 cells (Paper IV), all cell lines that express SV40 LTag and are reported to support JCPyV replication (43, 44, 118). We found that HEK 293TT and COS-7 cells were more permissive for JCPyV MAD-4 than SVG-A, but since HEK 293TT were not well attached to the wells, as also noted by others (248), we decided to use COS-7 cells for our investigations. Besides, COS-7 cells had previously been used for the study of CMX001 treatment of JCPyV replication (112).
Artesunate and antiviral targets in BKPyV and JCPyV replication

There are three main strategies used to combat viral infections: an antiviral drug can either target specific viral proteins (viral targets), cellular proteins (host targets) or modulate the immune response. In general, antiviral drugs targeting viral proteins ensure the specificity and safety but increases the possibility of resistance development, while antiviral drugs targeting cellular proteins and modulate immune response provoke less resistance development but have a higher risk of side effects (214, 244).

In Paper I, III and IV, we have seen that artesunate inhibits the replication of BKPyV and JCPyV, but what do we know about the target and the mechanism for this inhibition? Since BKPyV and JCPyV both have a small genome encoding only about 7 or 9 viral proteins, respectively, they have a limited number of potential viral targets for antiviral therapy. The main viral protein of interest for antiviral drug research has been the regulatory protein LTag. Some inhibitors of the ATPase activity of SV40 LTag (MAL2-11B, bisphenol A and hexachlorophene) are described (251, 252, 288), but a significant cytotoxicity limited their further investigations. Fluoroquinolones that selectively inhibit bacterial topoisomerase II/IV (11), have been shown to also inhibit BKPyV replication (92, 172, 223, 231, 259) and the mechanism for this inhibition was speculated to be inhibition of LTag helicase activity as suggested for SV40 LTag (8). However, in our previous study, the two fluoroquinolones ofloxacin and levofloxacin also reduced cellular DNA replication suggesting that the cellular topoisomerase II was also inhibited (259). In Paper I, we reported that artesunate at 10 µM reduced BKPyV LTag mRNA and protein expression by 30 and 75%, respectively. The reduction of early protein expression is similar to the effect of fluoroquinolones (259) but different from the effect of cidofovir, CMX001 and leflunomide (29, 30, 232).
Although we can conclude that artesunate reduces BKPyV LTag expression in RPTECs (Paper I), it is still undefined whether or not this is a direct or an indirect effect. Since all steps in the replication cycle are connected, inhibition of this early step inhibits all later steps thereby making it difficult to elucidate any effects of artesunate on late steps of the viral replication cycle. In BKPyV-infected HUCs, LTag expression was not specifically looked for but was probably not influenced by artesunate since the subsequent step, viral DNA replication, was unchanged (Paper III). These results suggest an effect of artesunate in HUCs on a step after viral genome replication. In Paper IV, where the effect of artesunate on JCPyV replication was investigated in COS-7 cells, we did not investigate the affected step(s) in the viral replication cycle since the host cells expressed high amounts of SV40 LTag which can initiate JCPyV replication (184), thereby complicating the picture.

Although the small viruses BKPyV and JCPyV depend on multiple cellular proteins for their replication, the limited knowledge on the replication cycle has delayed the recognition of potential cellular targets for antiviral therapy. R-Roscovitine, a cyclin-dependent kinase inhibitor which previously was found to have antiviral activity against a wide variety of herpes viruses, was shown to inhibit replication and cytopathic effect of JCPyV in vitro (214) and also suppress LTag expression during SV40 infection (139). R-Roscovitine has been evaluated in several Phase I and II studies where it has shown early signs of anti-cancer activity in approximately 240 patients and is now in Phase II testing for Cushings Disease (ClinicalTrials.gov NCT02160730) but has not yet been tested for viral infections. In our work, artesunate was found to induce cytostatic effects in RPTECs, HUCs and COS-7 cells (Paper I, III and IV). In RPTECs (Paper I) and COS-7 cells (Paper IV), the cellular DNA replication, which was monitored by BrdU incorporation, was reduced in a
concentration-dependent manner. In COS-7 cells, DNA replication was measured and found reduced already at 24 hours posttreatment (hpt) which may suggest that this step was directly affected by artesunate. For RPTECs (Paper I), the DNA replication was first measured at 72 hpt and the reduction found could therefore reflect the lower cell number as indicated by the real-time viability cell index. However, in Paper I, the cell cycle distribution of artesunate treated cells at 24 hpt was investigated in detail by flow cytometry and a 50% reduction of the cell population in S phase was found confirming the inhibition of cellular DNA replication. In RPTECs, HUCs and COS-7 cells (Paper I, III and IV), the metabolic activity was investigated by resazurin reduction and/or ATP content measurements and was found to be reduced in a concentration-dependent manner. In COS-7 cells (Paper IV), the reduction was measured already 24 hpt, which could indicate that the metabolic activity per cell was directly affected, while for RPTECs (Paper I) and HUCs (Paper III) metabolic activity was first measured at 72 hpt and based on the real-time viability cell index probably reflected a lower cell number rather than a reduced metabolism per cell.

Artesunate has shown antiviral activity against a wide variety of herpes viruses in vitro with no or little cytotoxicity (Table 2) (83, 155, 195, 247). Although the molecular mechanism for the anti-HCMV effect of artesunate is not completely understood, the NFκB or Sp1 dependent activation pathways seems to be inhibited and this inhibits the expression of HCMV immediate early genes and the viral DNA replication (17, 83, 84). We did look for NFκB expression in BKPyV-infected RPTECs but could not find an upregulation due to BKPyV infection as measured by western blot, and artesunate did not change the level of NFκB expression in mock-infected or BKPyV-infected RPTECs (unpublished results), suggesting that artesunate affects BKPyV replication
by a different mechanism. We have still not investigated the NFκB expression in JCPyV-infected cells.

**Artesunate: a possible future treatment for polyomavirus-associated diseases?**

Though we have found that artesunate can effectively inhibit BKPyV replication (Paper I and III) and JCPyV replication (Paper IV) in vitro, an important question is whether or not artesunate can be used for the treatment for polyomavirus-associated diseases. In addition to being used on millions of malaria patients, artesunate has recently been used to treat at least 14 patients with severe HCMV, HSV-2 or HHV-6B infections (105, 116, 169, 254, 258, 287) by oral or intravenous daily doses of up to 200 mg per day for up to 7 months. Seven of these patients recovered from their viral infection and in some of the unsuccessfully treated patients, a reduction of the viral load was seen. It should also be emphasized that for most of these patients, artesunate treatment was first started when other treatment options had failed. Since the EC$_{50}$ of artesunate for BKPyV replication in RPTECs (4.2 µM) and in HUCs (6.2 µM) (Paper I and III) and for JCPyV replication in COS-7 cells (3.0 µM) (Paper IV) is in the same range as reported for herpes viruses (2.16–7.21 µM) (Table 2), artesunate treatment of BKPyV and JCPyV infections seems to be a possible.

To treat PyVAN, artesunate and/or the active metabolite dihydroartemisinin, must reach the kidney tubular epithelial cells and the urothelial cells. After intravenous administration of artesunate at a dosage of 0.5-8 mg/kg to healthy volunteers and malaria patients, peak plasma concentration of artesunate in the range of 12.5-217 µM (4.8-83.3 µg/ml) have been reported (22, 142, 174, 208) indicating that the EC$_{50}$ concentration of artesunate at least is achievable in the plasma. The fate of artesunate in the kidneys is still not well known. Based on a rat model and one case study of an artesunate treated malaria patient, artesunate has been suggested to efficiently
passage through the glomerulus (49, 253). However, since only small amounts of artesunate and dihydroartemisinin are detected in the urine, this suggests that most of the filtered artesunate is reabsorbed from the tubules (143, 290, 294) and therefore should enter the tubular epithelial cells.

To treat PyVHC, artesunate and/or dihydroartemisinin needs to reach the affected sites in the urinary bladder. However, since the urothelium is multilayered, and the superficial transitional cells are hardest affected (210), we speculate that treatment from the apical i.e. urine side would be more efficient than via blood from the basolateral side. Although we have no information about artesunate uptake in urothelial cells in vivo, in the human intestinal epithelial cell line Caco-2, artesunate was found to enter the cells by passive diffusion (18) suggesting that artesunate could enter the cell from either side. Since only small amounts of artesunate and dihydroartemisinin are found in urine after intravenous administration, intravesical delivery of artesunate could possibly be one option. Although this has not been done before, the method has been used to administer other drugs to treat bladder cancer and cystitis (275). Moreover, intravesical delivery of cidofovir was used to treat 26 PyVHC patients (185) but while the patients improved clinically, a virologic response was missing. Our in vitro results suggest that the uptake of artesunate in HUCs (Paper III) is slower than uptake in RPTECs (Paper I) and COS-7 cells (Paper IV) but this can be different in vivo. Interestingly, it has been reported that absorption of instilled chemotherapeutic drugs can be enhanced by dimethyl sulfoxide (DMSO) (107). Since artesunate was dissolved in DMSO and since we found no disadvantageous effects of DMSO on HUCs viability (Paper III), intravesical delivery could be an option for the treatment of PyVHC.
To treat PML, artesunate and/or dihydroartemisinin needs to reach the affected sites in the brain. Very limited information is available about penetration of artesunate into the CNS. In a rat model, artesunate was found to cross the blood-brain barrier after intravenously administration of 5 mg/ml/kg, but only about 1% of the total dose was detected in the brain and even less in plasma (176, 294). However, as mentioned in Paper IV, in one study of six Plasmodium falciparum malarial patients treated intravenously with artesunate (66), no artesunate was detected in CSF but the concentration of dihydroartemisinin increased over time. Although this study has several limitations such as the number of patients included, that only one time point for each patient was examined and that the patients had malaria, it suggests that dihydroartemisinin is accumulated in the CSF and possibly can enter the brain. This is also supported by a study of Plasmodium berghei-infected mice (196), where a single dose of artesunate (32 mg/kg) improved survival and clinical signs of cerebral malaria. Although a large number of patients have been treated with artesunate without reported neurotoxicity, it will be important to monitor effects on the brain especially when doses exceeding the malaria defined doses are used. Interestingly, while TNF-α has been shown to induce the expression of LTag in IMR-32 cells and thereby contribute to JCPyV replication (211), artesunate was found to reduce the expression of TNF-α in the brain of mice with cerebral malaria (265). If this is also the case in human brains, this would be one mechanism for artesunate to reduce JCPyV replication.

If artesunate ever will be used for the treatment of PML for instance in HIV positive patients, it is important to be aware of possible interaction between artesunate and HIV antivirals as previously described in HIV positive malaria patients (158).
Future perspectives

As a strategy to improve the activity and the compliance of artemunate, two artemisinin units were connected into a stable dimeric molecule for single dose therapy (reviewed in (202)). Eighteen different artemisinin-derived dimers with potent anti-HCMV properties have been described (13, 122-124, 202). As for the monomer, the activity seems to be connected to their typical endoperoxide bridge (123). Of note, the artemisinin-derived dimers were found to have up to 500 fold higher activity against HCMV replication than the artemisinin monomers like artesunate (13) and could therefore be used in nanomolar (nM) concentrations. Interestingly, the artemisinin-derived dimers inhibited HCMV replication irreversibly and the inhibition was shown at an early stage of the viral replication cycle, on DNA replication and also on steps post DNA replication (124). Apparently, the dimers were not toxic to the host cells at the concentrations required to completely inhibit HCMV replication. In malaria infected mice, the dimers in combination with mefloquine increased survival significantly compared to treatment with the monomer artemether in combination with mefloquine (60). It would be very interesting to investigate the effect of artemisinin-derived dimers on BKPyV and JCPyV replication.

For some patients with HCMV infection, treatment with artesunate was given for 4 to 7 months (105). Probably the treatment period could have been shortened if the half-life of the drug was increased and the delivery improved. In the early 1990s, solid lipid microparticles were developed as a drug carrier system with sustained release (review in (53)). Recently, artesunate was formulated with lipid excipients as solid lipid microparticles (53). These microparticles showed good sustained release properties, could be administrated once daily and showed superior antimalarial properties over normal artesunate administration in a mouse model (53). Since our in vitro testing
showed that artesunate had rapid but reversible effect on BKPyV replication in RPTECs (Paper I, Figure 5A), the microparticles keeping a more constant artemisinin concentration could be an attractive delivery vehicle and should be considered for future studies.

Combination therapy is now increasingly used for the treatment of cancer, tuberculosis, HIV, hepatitis C virus and malaria but has not been common for treatment of polyomavirus-associated diseases. Importantly, the drug combination must have synergistic effect. Recently, the Bliss model (32) was suggested to study the synergistic, antagonistic or additive effects of anti-HCMV drugs in vitro (48). This model could be used to study the anti-BKPyV and anti-JCPyV effect of artemisinin in combination with other drugs. The effect of several drugs like fluoroquinolones, cidofovir, leflunomide and CMX001 on BKPyV replication in RPTECs (29, 30, 232, 259) and CMX001 on JCPyV replication in primary human astrocytes (112) have previously been characterized. Based on these results, the known organ distribution and safety profile, we would like to investigate the combination of artemisinin and fluoroquinolones on BKPyV replication; and artemisinin and CMX001 on JCPyV replication. If synergistic effects were found without increased cytotoxicity, the efficacy of artemisinin treatment could thereby be further increased.
Main conclusions

We have found that artesunate can inhibit the replication of BKPyV and JCPyV in cell culture, and that this inhibition is closely connected to cytostatic effects suggesting a mechanism affecting cellular proteins. We have performed our studies in appropriate cell model systems for BKPyV but a less relevant cell model for JCPyV since authentic cells were not available. However, since the EC$_{50}$ found was similar for BKPyV and JCPyV and since these viruses are closely related, we think that also JCPyV replication in other cells will be inhibited by artesunate. Taken together with the EC$_{50}$ found for herpes viruses, the described organ distribution and safety of artesunate, we think that artesunate or a modified form of artesunate or artesunate in combination with one of the adjunctive drugs in use could be beneficial for treatment of PyVAN, PyVHC and PML. By pursuing artesunate as an antiviral treatment option, we can greatly benefit from the ongoing artesunate research and the clinical studies on malaria patients that are taking place. Finally, carefully designed clinical studies have to be performed in patients suffering from PyVAN, PyVHC and PML to answer if artesunate is of benefit or if this is just one more poorly effective drug.
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