A Retrograde Trafficking Inhibitor of Ricin and Shiga-Like Toxins Inhibits Infection of Cells by Human and Monkey Polyomaviruses

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ABSTRACT **Polyomaviruses are ubiquitous pathogens that cause severe disease in immunocompromised individuals. JC polyomavirus (JCPyV) is the causative agent of the fatal demyelinating disease progressive multifocal leukoencephalopathy (PML), whereas BK polyomavirus (BKPyV) causes polyomavirus-induced nephropathy and hemorrhagic cystitis. Vaccines or antiviral therapies targeting these viruses do not exist, and treatments focus on reducing the underlying causes of immunosuppression. We demonstrate that retro-2^{cycl}, an inhibitor of ricin and Shiga-like toxins (SLTs), inhibits infection by JCPyV, BKPyV, and simian virus 40. Retro-2^{cycl} inhibits retrograde transport of polyomaviruses to the endoplasmic reticulum, a step necessary for productive infection. Retro-2^{cycl} likely inhibits polyomaviruses in a way similar to its ricin and SLT inhibition, suggesting an overlap in the cellular host factors used by bacterial toxins and polyomaviruses. This work establishes retro-2^{cycl} as a potential antiviral therapy that broadly inhibits polyomaviruses and possibly other pathogens that use retrograde trafficking.**

IMPORTANCE **The human polyomaviruses JC polyomavirus (JCPyV) and BK polyomavirus (BKPyV) cause rare but severe diseases in individuals with reduced immune function. During immunosuppression, JCPyV disseminates from the kidney to the central nervous system and destroys oligodendrocytes, resulting in the fatal disease progressive multifocal leukoencephalopathy. Kidney transplant recipients are at increased risk of BKPyV-induced nephropathy, which results in kidney necrosis and loss of the transplanted organ. There are currently no effective therapies for JCPyV and BKPyV. We show that a small molecule named retro-2^{cycl} protects cells from infection with JCPyV and BKPyV by inhibiting intracellular viral transport. Retro-2^{cycl} treatment reduces viral spreading in already established infections and may therefore be able to control infection in affected patients. Further optimization of retro-2^{cycl} may result in the development of an effective antiviral therapy directed toward pathogens that use retrograde trafficking to infect their hosts.**

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Human polyomaviruses are widespread pathogens that establish persistent lifelong infections in their hosts (1, 2). JC polyomavirus (JCPyV) and BK polyomavirus (BKPyV) establish persistent infections early in life and chronically infect kidney cells, urinary tract cells, tonsillar stromal cells, and bone marrow-derived cells (3–6). The seroprevalences of JCPyV and BKPyV are 50 and 80%, respectively (7). It is likely that JCPyV and BKPyV persistently replicate at low levels, as virus is sporadically detected in the urine of 30% of the individuals tested (8).

Under conditions of immunosuppression, such as AIDS or immunomodulatory therapy, increased replication of JCPyV results in dissemination of the virus to the central nervous system (5). Lytic infection of oligodendrocytes by JCPyV results in the fatal demyelinating disease progressive multifocal leukoencephalopathy (PML) (9). The incidence of PML in AIDS patients is between 3 and 5%, and the incidence in patients receiving immunomodulatory therapies is between 0.2 and 0.4% (5). BKPyV-associated disease is most often seen in the context of renal transplantation, where immunosuppressive therapies result in increased replication of BKPyV in the transplanted kidney, leading to hemorrhagic cystitis and polyomavirus-induced nephropathy (PVN) (10). The incidence of PVN in transplant recipients can be as high as 10%, often resulting in loss of the transplanted kidney (11). There are no effective antiviral therapies to combat polyomavirus infection.

Despite being structurally simple, polyomaviruses use a complex and incompletely understood entry process to effect transport to the nucleus, where viral transcription and DNA replication occur. After binding to cellular receptors on the cell surface, polyomaviruses enter the classical endocytic pathway (12–14). From early or late endosomes, all of the polyomaviruses studied to date undergo transport to the endoplasmic reticulum (ER), where they interact with ER chaperones to partially disassemble their capsid, resulting in retrotranslocation of the virion into the cytosol (15–19). Despite the importance on ER trafficking, the specific

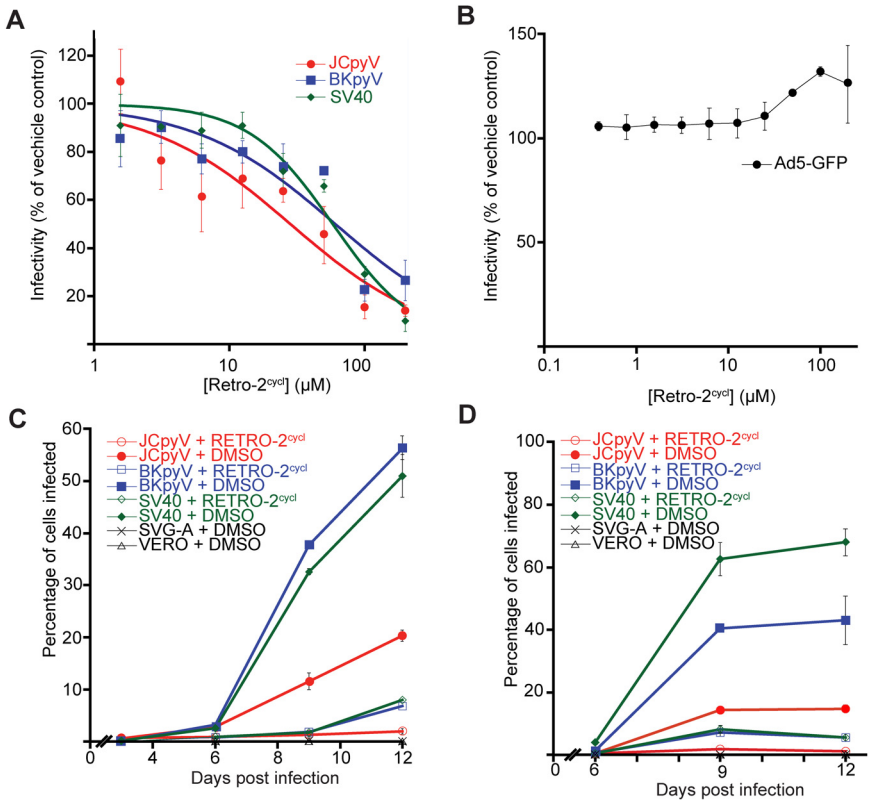

FIG 1 Retro-2^{cycl} prevents infection with three polyomaviruses. (A) Dose-dependent effect of retro-2^{cycl} treatment on infection. Cells were preincubated with the indicated concentrations of retro-2^{cycl} prior to inoculation with virus. Infections were scored and normalized to a DMSO-treated sample. (B) Retro-2^{cycl} does not block infection with adenovirus pseudovirus. Vero cells were pretreated with equivalent concentrations of retro-2^{cycl} prior to infection with an Ad5-GFP pseudovirus. Cells were scored and normalized to a DMSO-treated sample. (C) Retro-2^{cycl} prevents virus spreading in a multicycle growth assay. Cells were infected with virus for 72 h. Cells were then maintained in 0.1 mM retro-2^{cycl}. Cells were scored for infection every 3 days. (D) Retro-2^{cycl}-treated cultures release less infectious virus into culture medium. Tissue culture medium was harvested every 3 days and used to infect naive cells not treated with retro-2^{cycl}. The data represent the mean of three replicates, and error bars indicate the standard deviation.

host cellular machinery used to promote ER targeting of virions remains unclear.

In this study, we demonstrate that the small molecule 2-{[(5-methyl-2-thienyl)methylene]amino}-*N*-phenylbenzamide (retro-2^{cycl}) potently inhibits the infection of tissue culture cells by JCPyV, BKPyV, and the closely related primate virus simian virus 40 (SV40). Retro-2^{cycl} was previously identified by high-throughput screening for small molecules that inhibit the intoxication of host cells by ricin, Shiga-like toxins (SLTs), and the cholera toxin B subunit (CTxB) (20). Retro-2^{cycl} inhibits retrograde trafficking from endosomes to the Golgi apparatus, thus preventing the intoxication of host cells. Rather than binding to the toxins, retro-2^{cycl} interferes with retrograde trafficking of cargo by interaction with an unidentified cellular host factor. We demonstrate that retro-2^{cycl} inhibits polyomavirus infection by inhibiting ER transport, suggesting that polyomaviruses, ricin, SLTs, and CTxB share a dependency on similar retro-2^{cycl} sensitive host factors for successful intracellular transport. Recently, we found that retro-2^{cycl} also inhibits the infection of cells by human papillomaviruses (21). Further optimization of this compound may result in the development of effective antiviral compounds that inhibit the infection of cells by viruses requiring ER transport for infection.

RESULTS

Retro-2^{cycl} inhibits polyomavirus infection in a dose-dependent manner. To determine whether retro-2^{cycl} inhibits infection by polyomaviruses, we pretreated permissive cells with retro-2^{cycl} and infected them with JCPyV, BKPyV, or SV40 (20). Retro-2^{cycl} treatment resulted in a dose-dependent decrease in infected cells compared to a vehicle control, with calculated 50% effective concentrations of 28.4, 61.2, and 58.6 μM for JCPyV, BKPyV, and SV40, respectively (Fig. 1A). As a control, we pretreated Vero cells with retro-2^{cycl} and inoculated them with a green fluorescent protein (GFP)-expressing adenovirus (Ad5-GFP), which is known not to require retrograde trafficking (22). Retro-2^{cycl} treatment did not inhibit adenovirus-mediated transduction, suggesting that the effect of retro-2^{cycl} on infection is specific to virions that undergo retrograde trafficking (Fig. 1B). We additionally infected a panel of cell lines with JCPyV pseudovirus expressing a luciferase reporter gene. Retro-2^{cycl} exhibits similar levels of inhibition in the brain-derived SVG-A, HFGT, and POJ19 cell lines, as well as the kidney-derived Vero, Cos-7, 293A, and 293FT cell lines, indicating that retro-2^{cycl} is protective of numerous cell lines derived from multiple organs (see Fig. S1 in the supplemental material). Retro-2^{cycl} exhibited low cellular toxicity at protective levels (see Fig. S2).

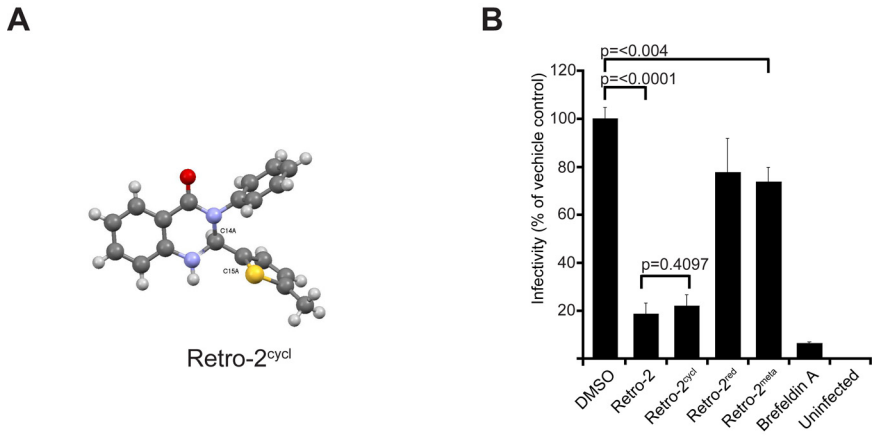

FIG 2 Structure of retro-2^{cycl} and inhibitory activities of retro-2 analogs. (A) The structure of retro-2^{cycl} was solved by X-ray diffraction, and it was verified to be a DHQ. (B) Retro-2^{cycl} protects cells from polyomavirus infection. Cells were pretreated with retro-2, the indicated analog, the vehicle control, or BFA (71 nM, 20 ng/ml) and infected with JCPyV. Infected cells were scored and normalized to the vehicle-treated control. The data represent the average of triplicate samples. Error bars indicate the standard deviation.

Retro-2^{cycl} is able to reduce viral spreading in established tissue culture infections. Since most individuals are persistently infected with JCPyV or BKPyV prior to immunosuppression, we asked whether retro-2^{cycl} could prevent viral spreading in established tissue culture infections. Cells were infected at a low multiplicity of infectivity (MOI) of 0.01. Following one round of productive infection, 100 μM retro-2^{cycl} was added to these cells and maintained during the course of the experiment. Treatment of cells resulted in a significant reduction of infected cells compared to the vehicle control, and this effect was most striking at 12 days postinfection, where retro-2^{cycl} diminished the spreading of SV40 (84% inhibition), BKPyV (89%), and JCPyV (90.5%) (Fig. 1C). To examine whether the treatment of these cultures with retro-2^{cycl} inhibited virion production, supernatants from each time point were used to reinfect naive cells that were not retro-2^{cycl} treated. Cultures that were previously treated with retro-2^{cycl} produced significantly less infectious virions (Fig. 1D), demonstrating that retro-2^{cycl} decreases the cell-to-cell spreading of polyomaviruses in previously infected cultures.

The bioactive compound is a DHQ derivative of retro-2. After showing that commercially purchased retro-2 inhibits polyomavirus infection, we chemically synthesized the compound by a previously reported method in order to facilitate subsequent investigations (23). Condensation of 2-aminobenzanilide with 4-methyl-2-thiophencarboxaldehyde in the last step of synthesis yielded a mixture of two products, both having the expected molecular weight of retro-2 (see Fig. S3A in the supplemental material). The two compounds were separated and independently characterized. One product was retro-2, as indicated by a characteristic singlet at 11.0 ppm for the imine proton, as determined by nuclear magnetic resonance analysis (data not shown). The second product was revealed to be a dihydroquinazolinone (DHQ) by X-ray diffraction crystallography and is termed retro-2^{cycl} (Fig. 2A). The spectroscopic data for the compound purchased from ChemBridge were identical to those for the DHQ derivative and not to those of the structure reported for retro-2 (data not shown). To test the inhibitory effects of both compounds on JCPyV infection, SVG-A cells were incubated with 100 μM retro-2 or retro-2^{cycl} and challenged with JCPyV. Surprisingly, both retro-2 and retro-2^{cycl} inhibited polyomavirus infection with similar efficacies (Fig. 2B).

Imine species similar in structure to retro-2 are commonly invoked as mechanistic intermediates in the formation of DHQ (24–28), which suggests that the two chemical species could interconvert in the infectivity assay, thus accounting for their similar biological activities. Accordingly, we found that treatment of retro-2 with scandium(III) trifluoromethanesulfonate in methanol resulted in rapid conversion to the DHQ (data not shown). In aqueous medium, retro-2 most likely cyclizes into a DHQ as well. During the preparation of this report, another group reported that retro-2 slowly cyclizes in methanol and named this compound "Retro-2^{cycl}" (29).

It was still unclear whether the biologically active compound was an imine or a DHQ. Treatment of retro-2^{cycl} with sodium cyanoborohydride in methanol slowly produced a reduced species (retro-2^{red}), which indicated that cyclization is reversible and that retro-2 and retro-2^{cycl} exist in equilibrium (see Fig. S3B in the supplemental material). However, despite their structural similarities, retro-2^{red} is significantly less active than retro-2 (Fig. 2B). We also prepared a retro-2 regioisomer wherein the carboximide and imine moieties are *meta* substituted (retro-2^{meta}), therefore precluding cyclization (see Fig. S3C). This compound was also significantly less active and served as a useful negative control in subsequent experiments. Together, the lack of biological activity intrinsic to retro-2^{red}, as well as retro-2^{meta}, leads us to the conclusion that the chemical species responsible for inhibition of polyomavirus infection is, in fact, the DHQ, retro-2^{cycl}.

Retro-2^{cycl} inhibits polyomavirus infectivity at early time points during infection. We hypothesized that retro-2^{cycl} inhibited retrograde trafficking of polyomavirus to the ER. SVG-A or Vero cells were synchronously infected with JCPyV, BKPyV, or SV40, and retro-2^{cycl} was added to cells at the indicated time points. The results show that the addition of retro-2^{cycl} at time points up to 4 h postinfection (hpi) significantly reduces infectivity, with a progressive loss of its inhibitory effect from 6 to 18 hpi (Fig. 3A). These kinetics are consistent with previous reports showing that polyomaviruses colocalize with ER markers at 6 to 16 hpi, demonstrating that the protective effect of retro-2^{cycl} is lost

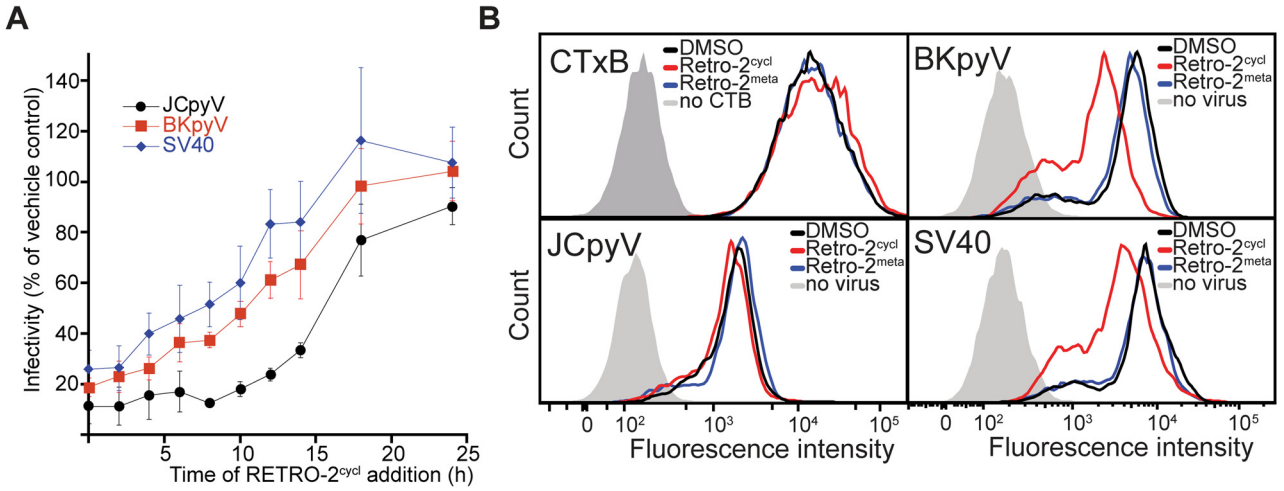

FIG 3 Retro-2^{cycl} inhibits polyomavirus infectivity at early time points during infection. (A) Cells were chilled prior to incubation with JCPyV, BKPyV, or SV40 to synchronize infections, and retro-2^{cycl} (0.1 mM) was added at the indicated time points. After 72 h, infected cells were scored and normalized to the vehicle control. The data represent the mean of three replicates, and error bars indicate the standard deviation. (B) Effect of retro-2^{cycl} on virus binding to cells. Cells were detached and pretreated with the vehicle control or retro-2^{cycl} for 30 min at 37°C. Cells were then inoculated with Alexa Fluor 633-labeled JCPyV, BKPyV, SV40, or CTxB for 1 h on ice. Samples were then washed and read by flow cytometry. CTB, CTxB.

following time points consistent with localization to the ER (13–15, 30). To rule out an effect on virus binding, we treated cells with retro-2^{cycl} or retro-2^{meta} and measured the binding of labeled virus or cholera toxin to cells by flow cytometry. Treatment of the cells with retro-2^{cycl} or retro-2^{meta} had no effect on the binding of CTxB or JCPyV to cells but slightly reduced the binding of BKPyV and SV40 (Fig. 3B). The reduction of SV40 binding is not due to a reduction of cell surface receptor expression, as CTxB binding to GM1, also the receptor for SV40, was not reduced (Fig. 3B). Retro-2^{cycl} also does not interact directly with either SV40 or BKPyV, as incubation of retro-2^{cycl} with either virus does not decrease the ability to bind to cells (see Fig. S4 in the supplemental material). Retro-2^{cycl} also had no effect on the endocytosis of JCPyV, BKPyV, SV40, or CTxB (see Fig. S5).

Retro-2^{cycl} reduces retrograde trafficking of polyomaviruses to the ER. Since retro-2^{cycl} prevents intracellular trafficking of ricin, SLTs, and CTxB, we sought to determine whether treatment of cells with retro-2^{cycl} would interfere with the delivery of virions to the ER. Cells were preincubated with retro-2^{cycl} or a vehicle control and then inoculated with virus. Cells were then fixed at 8 hpi and immunostained for the ER protein PDI (protein disulfide isomerase) and VP1. To determine the colocalization of these two proteins, we used a proximity ligation assay (PLA) that generates a fluorescent signal only when the target proteins are within 40 nm of each other. Colocalization of polyomaviruses and PDI was readily detectable in cells treated with the vehicle control (Fig. 4A and B). In contrast, treatment of cells with brefeldin A (BFA) or retro-2^{cycl} significantly reduces the PLA signal between VP1 and PDI, demonstrating that virion ER transport is inhibited (Fig. 4B). When fluorescently labeled BKPyV is added to cells in the presence of retro-2 ^{cycl}, redistribution of virions from a reticular, ER-type pattern toward the cell periphery is observed, further demonstrating that retro-2^{cycl} alters virion transport (see Fig. S6 in the supplemental material).

Retro-2^{cycl} prevents exposure of the viral minor capsid proteins. Upon trafficking to the ER, polyomaviruses interact with host cell chaperones to isomerize their interpentameric disulfide bonds and expose the previously sequestered minor capsid protein VP2 (18, 19, 31, 32). Exposure of VP2 can therefore further verify whether ER transport is inhibited in retro-2^{cycl}-treated cells. We inoculated cells with JCPyV, BKPyV, or SV40 in the presence of 100 μM retro-2^{cycl} or the vehicle control. In cells treated with the dimethyl sulfoxide (DMSO) control, discrete puncta corresponding to VP2 were visualized at 10 hpi in a perinuclear region of the cells (Fig. 5A), similar to previously published reports (19). In contrast, treatment of cells with BFA results in a significant reduction in the number of cells with VP2 exposed, demonstrating that ER transport is required to expose VP2. Treatment of cells with retro-2^{cycl}, but not poorly neutralizing retro-2^{meta}, resulted in a significant reduction in the proportion of cells exposing VP2 to levels similar to those of cells treated with BFA (Fig. 5A and C). These puncta colocalize with the ER rather than other organelles such as lysosomes, a result that is consistent with previous studies (Fig. 5B) (19). We also observed a decrease in VP2 and PDI association by PLA after retro-2^{cycl} treatment, further demonstrating that VP2 is exposed in the ER (see Fig. S7 in the supplemental material). Thus, retro-2^{cycl} treatment of cells reduces ER trafficking and, as a consequence, prevents exposure of the minor capsid proteins of JCPyV, BKPyV, and SV40.

DISCUSSION

We demonstrate that the small molecule retro-2^{cycl}, a recently described inhibitor of bacterial intoxication, inhibits infection by three polyomaviruses. Retro-2^{cycl} inhibits polyomavirus infection in a manner similar to its effects on ricin and Shiga-like toxins, blocking retrograde transport to the ER or Golgi compartment (20). This effect appears to be specific for viruses that use retrograde transport, as transduction of cells by a GFP-expressing adenovirus is not inhibited by retro-2^{cycl}. This work demonstrates that retro-2^{cycl} is not only an effective antiviral compound but will also aid in the further delineation of the endocytic pathways used by polyomaviruses to target the ER.

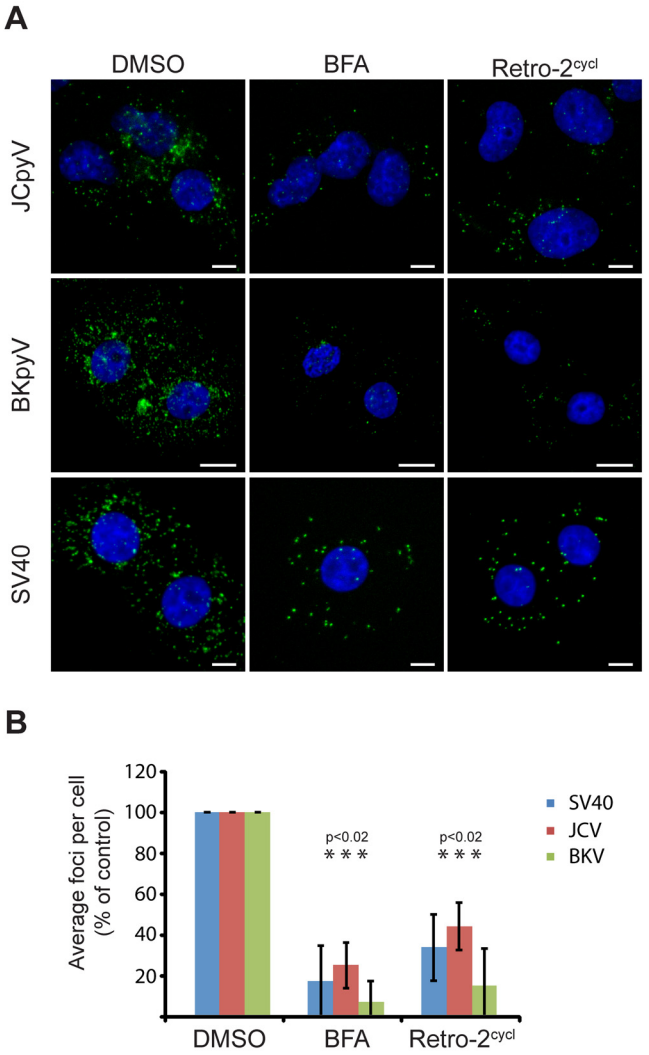

FIG 4 Retro-2^{cycl} inhibits polyomavirus ER trafficking. (A) Colocalization was assessed with a PLA. Error bars denote the standard deviation. (B) Cells were pretreated with the indicated drug (500 ng/ml BFA or 0.1 mM retro-2^{cycl}) for 0.5 h prior to inoculation with JCPyV, BKPyV, or SV40 at an MOI of 100. Cells were incubated for 8 h with the indicated drugs, fixed, and permeabilized. Cells were then stained with a mouse monoclonal antibody to PDI and a rabbit polyclonal antibody to VP1 prior to detection by PLA. Fluorescent foci indicate areas of colocalization. BKV, BKPyV.

A critical step in the infectious entry of polyomaviruses is ER transport, and we show that retro-2^{cycl} significantly reduces the transport of virions to the ER (32, 33). Since virions cannot interact with ER-resident chaperones in retro-2^{cycl}-treated cells, necessary uncoating steps are inhibited, as evidenced by a lack of exposure of the minor capsid protein VP2 in retro-2^{cycl}-treated samples. All of the polyomaviruses studied to date undergo ER transport, and in recent years, nine new human polyomaviruses have been discovered (34). Several of these newly discovered viruses are associated with human diseases, including Merkel cell polyomavirus, which is the causative agent of the fatal cancer Merkel cell carcinoma (35). Since retro-2^{cycl} is protective against JCPyV, BKPyV, and SV40, it is likely that this compound will inhibit the replication of these new polyomaviruses and will be a useful tool in verifying whether these new polyomaviruses target

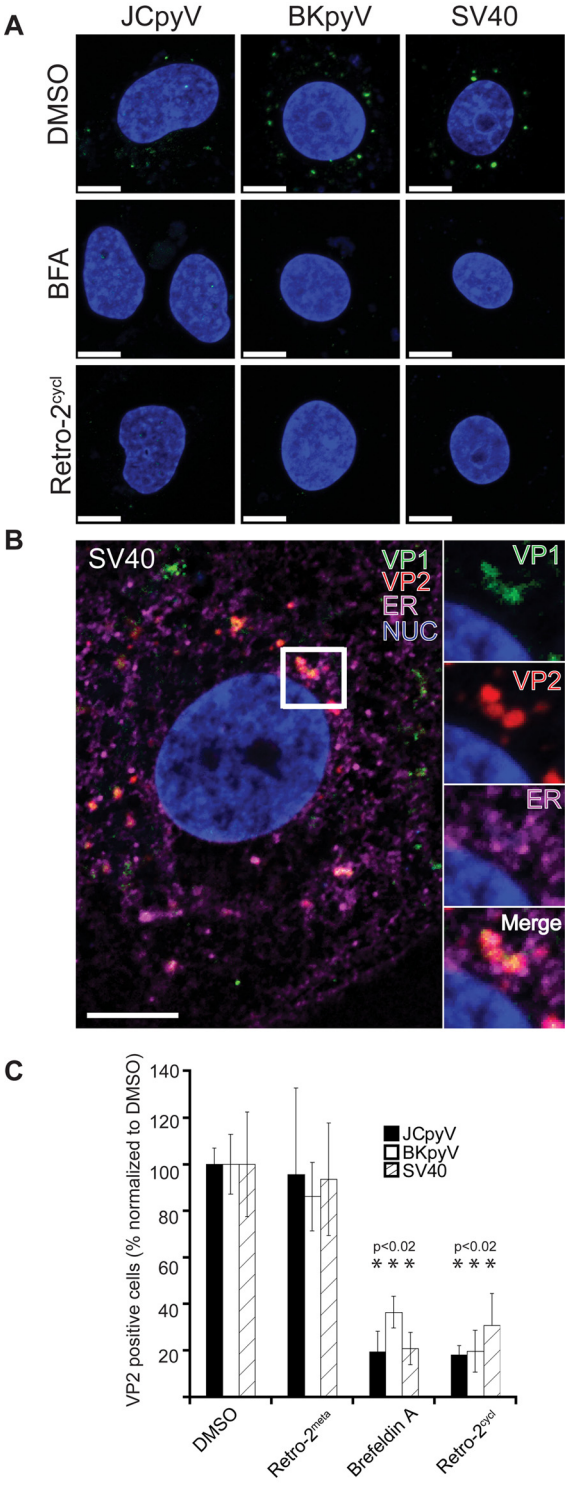

FIG 5 Retro-2^{cycl} inhibits VP2 exposure of polyomaviruses. (A) VP2 is exposed at late time points during infection. Cells were pretreated with the indicated drugs and then inoculated with JCPyV, BKPyV, or SV40 at an MOI of 10 for 10 h before fixation and staining for VP2. VP2 puncta are green, and nuclei are blue. Scale bars, 10 μm. (B) VP2 is exposed in the ER. Cells were incubated with SV40 for 10 h, fixed, and then stained for VP1 (green), VP2 (red), and PDI (purple), and the nuclei were stained with BOBO-3 (blue). On the right, enlargements of the boxed area of the fluorescence micrograph show individual antibody staining. (C) Quantitation of panel A. Cells from triplicate samples were scored for the presence of VP2. Error bars show the standard deviations.

the ER for productive infection. We also found that pretreatment of cells with retro-2^{cycl} had no effect on the binding of JCPyV or CTxB to cells but did slightly reduce the binding of BKPyV and SV40. This is unlikely to be due to reductions in cell surface receptor expression, as the SV40 receptor, GM1, was not reduced, as evidenced by the binding of CTxB. This was also not due to direct binding of retro-2^{cycl} to the virions, as incubation of labeled viruses with retro-2^{cycl} did not reduce binding. Finally, treatment of the cells did not prevent any of these viruses from entering the cell. The major effect is at the level of intracellular trafficking to the ER.

BFA is another small molecule that has been reported to inhibit the ER accumulation of polyomaviruses (30, 31, 36–38). However, BFA is highly cytotoxic to cells, making this molecule less appealing for the development of antiviral or antitoxin therapies (39). Additionally, BFA treatment rapidly alters the morphology of the Golgi apparatus, inhibits endosomal maturation, and inhibits protein secretion, demonstrating that this compound elicits numerous effects besides inhibiting retrograde trafficking (40–43). Conversely, retro-2^{cycl} does not alter cell compartment morphology and is well tolerated when administered to mice (20). Thus, retro-2^{cycl} is likely the first small-molecule inhibitor of polyomavirus infectivity that shows promise as a potential antiviral therapy.

We also show that the biologically active chemical species of retro-2 is a DHQ derivative of retro-2 and not an imine, as was originally reported (20). While we were completing these studies, another group confirmed that retro-2 is converted to a DHQ (29). With the correct structure of the retrograde transport inhibitor now established, we can consider the medicinal chemistry optimization of retro-2^{cycl} as a potential drug lead.

The inhibitory effect of retro-2^{cycl} is strikingly similar to the effect seen on ricin toxin and Shiga-like toxins, where retro-2^{cycl} treatment prevents endosome-to-Golgi apparatus trafficking and, as a consequence, also inhibits ER trafficking (20). This suggests that there may be an overlap of the cellular proteins used by toxins and polyomaviruses to effect ER transport. However, there are likely significant differences in the kinetics or pathways used by polyomaviruses and bacterial toxins to target the ER, since ricin and Shiga-like toxins rapidly traffic to the Golgi apparatus (44), an association that has yet to be identified for any polyomavirus. This suggests that the cellular host factors targeted by retro-2^{cycl} may be involved in multiple retrograde trafficking pathways, that only a small proportion of virions traffic to the Golgi compartment, or that polyomaviruses may rapidly traffic through the Golgi complex prior to ER accumulation. SLTs, CTxB, and some polyomaviruses bind to glycolipids and may therefore provide a rationale for how this compound inhibits trafficking (14, 45–50). However, whereas numerous host cellular transport factors are known to promote endosome-to-Golgi apparatus transport of ricin toxins and SLT, such as the retromer complex, syntaxin 5 and 16, EpsinR, Rab6a, and clathrin, the roles of these factors in polyomavirus entry are not known (51–53, 57, 58). Additionally, transport to late endosomes is known to be important for polyomaviruses, but it is not known whether cellular retrograde transport factors involved in transport from late endosomes, such as Rab9 and Tip47, are important for polyomavirus infectivity. Future work examining the role of these host factors in polyomavirus infection and determining what cellular host factor retro-2^{cycl} binds will aid our understanding of how polyomaviruses and toxins undergo retrograde trafficking.

It is unlikely that retro-2^{cycl} binds to polyomaviruses directly and therefore decreases the likelihood of escape mutations, since infectious mutants would have to use alternate trafficking pathways to enter cells. Since the majority of people are persistently infected with JCPyV and BKPyV (2, 7, 54–56), the ability of retro-2^{cycl} to reduce the spreading of JCPyV, BKPyV, and SV40 in established infections suggests that these compounds may control viral dissemination in previously infected individuals. Further optimization of retro-2^{cycl} may result in effective antiviral therapies to treat or prevent diseases caused by human polyomaviruses or other pathogens that use retrograde trafficking during infection.

MATERIALS AND METHODS

Cells, viruses, plasmids, and antibodies. For all of the details of the cells, viruses, plasmids, and antibodies used in this study, see Text S1 (Materials and Methods) in the supplemental material.

Retro-2^{cycl} inhibition of infection. For all of the details of the infection studies described here, including dose-dependent inhibition of infection by retro-2^{cycl}, time course experiments, multicycle growth assays, and cell tropism experiments, see Text S1 (Materials and Methods) in the supplemental material.

Flow cytometry. For all of the details of the flow cytometry done in this study, including scoring of viral infectivity and binding assays, see Text S1 (Materials and Methods) in the supplemental material.

Viability assays. For all of the details of the viability assays done in this study, see Text S1 (Materials and Methods) in the supplemental material.

Virus purification and labeling. For all of the details of the virus purification and labeling done in this study, as well as details of pseudovirus production, see Text S1 (Materials and Methods) in the supplemental material.

Retro compounds. For all of the details of the synthesis of retro-2^{cycl} and its chemical derivatives, as well as X-ray crystallography and the deposition of X-ray structures, see Text S1 (Materials and Methods) in the supplemental material

Microscopy. For all of the details of the virus purification and labeling done in this study, as well as details of PLAs, see Text S1 (Materials and Methods) in the supplemental material.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00729-13/-/DCSupplemental.

Text S1, DOCX file, 0.1 MB.
Figure S1, TIF file, 0.5 MB.
Figure S2, TIF file, 0.9 MB.
Figure S3, TIF file, 0.9 MB.
Figure S4, TIF file, 1.2 MB.
Figure S5, TIF file, 1.2 MB.
Figure S6, TIF file, 7 MB.
Figure S7, TIF file, 1.5 MB.

ACKNOWLEDGMENTS

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