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ORIGINAL ARTICLE

Suppression of early and chronic BK polyoma virus replication by mycophenolic acid in Vero cells

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Summary

Consensus is lacking about which immunosuppressant agents potentiate BK virus infection. The effects of mycophenolic acid (MPA) were investigated in BK virus (BKV)-infected Vero E6 cells. MPA (1–16 mg/l) exhibited a dose-dependent anti-viral effect (10^1–10^4 fold reduction in BKV DNA copies/ml) in supernatant, similar to cidofovir (2.5–25 mg/l). This effect was observed for early and persistent infection, and infection with noncoding control region (NCCR) rearranged BKV. MPA reduced BKV DNA copies/ml by >1 log after day 14 in three patient isolates before and after NCCR rearrangement, and in cells. MPA reduced total cellular protein levels, consistent with an anti-metabolite effect without increased cytopathic activity. BKV infection was associated with a transient, significant reduction of collagen 1A1 on day 7 but not on days 14, 21, and 28 or in the presence of MPA. Reduction of alpha smooth muscle actin mRNA was observed only in the BKV + MPA group, and only on day 7. There was no significant alteration of heat shock protein 47 or transforming growth factor-β mRNA expression. These *in vitro* data suggest that MPA may have a protective, anti-viral effect in BKV-infected renal tubular cells with an anti-viral response. Maintaining, or even increasing, the MPA dose should be evaluated for reduction of BKV viremia levels.

Introduction

Polyoma virus-associated nephropathy (PVAN) is a growing cause for concern in the kidney transplant population. Typically caused by reactivation of the BK polyoma virus (BKV) [1], PVAN occurs in 1–10% of kidney transplant recipients [1–3] and ultimately results in graft loss in approximately half of all cases [1]. Cases of PVAN have also been reported following cardiac, pancreas and liver transplantations [4–8]. Although many risk factors have been proposed for PVAN [1], increasing intensity of immunosuppression is widely believed to exert an important influence [9–12]. Results from a prospective trial [13] and retrospective studies [14,15] suggest that triple therapy with tacrolimus, mycophenolic acid (MPA), and steroids may be associated with a particularly high risk of PVAN. Clinical data concerning a specific effect of MPA on the risk of BK polyoma virus infection or BK-related PVAN, however, are unconvincing. While the majority of cases of PVAN reported in the literature have occurred in patients receiving mycophenolate mofetil (MMF) [1,15,16], others have been reported in MPA-free patients [1] and there are preliminary data to suggest that MMF dose is not a significant risk factor for BKV-related PVAN [17]. Recently, a prospective study in 200 renal transplant recipients randomized to tacrolimus or cyclosporine showed no positive correlation between risk of BK viruria or viremia and treatment with MMF [13]; in this study, 38% of the 88 patients who were treated with MMF had BK viruria, as compared with an incidence of 35% in the total study population. While the highest incidence of viruria was seen in patients receiving tacrolimus and MMF, the lowest incidence was observed with cyclosporine and MMF, a finding that does not seem to support a specific

correlation between MPA therapy and increased risk of BKV infection.

Preliminary *in vitro* data have suggested that MPA may exert an anti-viral effect against HIV [18–20], hepatitis B virus [21–23] and herpes viruses [24], although conflicting findings have also been reported [25,26]. To date, the effect of MPA on BKV replication *in vitro* has not been examined. We have recently established a relevant kidney cell line to evaluate BKV replication in the presence of immunosuppressive agents [27,28] and report here our results concerning the effect of MPA on primary and chronic BKV infection.

Materials and methods

Cell culture system

A detailed description of the cell culture system has been published previously [27]. In summary, Vero E6 cells of Green monkey kidney cell origin were cultured [alpha minimal essential medium (αMEM) with Earle's salts (Invitrogen Corp, Carlsbad, CA, USA) and 10% fetal calf serum (FCS; Invitrogen Corp) and Pen-Strep (100 U penicillin, 100 μg streptomycin/ml; Invitrogen Corp)] and infected with BKV when the culture attained 70–90% confluency. After infection, FCS was reduced to 0.1% for chronic culture and media was exchanged weekly. The merits of this system are as follows: (i) Vero cells can accommodate several DNA viruses and achieve confluence readily; (ii) cell growth can be altered by the percentage of FCS; (iii) BKV-infected Vero cells are amenable to culture up to 12 weeks when copies of BKV DNA in the supernatant are $<10^4$ copies/ml; (iv) establishment of chronic BKV infection is associated with up to 10^9–10^{10} BKV DNA copies/ml (often by week 6) and this is associated with transformation of BKV from patient isolate to a non-coding control region (NCCR)-rearranged genotype (confirmed by gel electrophoresis); (v) BKV infection can be affected by inclusion of drugs in the supernatant; and (vi) evolution to chronic low-level BKV infection (10^2–10^4 copies/ml) after primary infection peak is influenced by BKV isolate, drug administration and cell passage number. Both control and cyclosporine A (CsA)-treated cells were monitored weekly for cytopathic effect (CPE). Supernatant (SN) lactic dehydrogenase levels were measured infrequently as a surrogate marker for cell lysis to correlate with our observation of CPE. MPA (Sigma-Aldrich Co., Oakville, ON, Canada), dissolved in methanol, was administered in the dose range 1–16 μg/ml. MPA concentration in the supernatant and cell suspensions was measured by the clinical reference laboratory at the QEII Health Sciences Center, Halifax, Nova Scotia, Canada.

BKV infection and monitoring

Three different patient isolates of pathogenic BKV strains were used in chronic culture (VJ, JB and LH), each of which has been assessed previously for infectivity and performance profile [27]. Every 7 days, the media were exchanged and a 200 μl aliquot was reserved for DNA extraction. The media were removed at the end of each experiment and cells were trypsinized (500 μl 0.25% Trypsin; Invitrogen Corp). Five-hundred microliters of αMEM (2% FCS) was added to each well, and a 200 μl aliquot was extracted for cellular fraction of BKV, and a 10 μl aliquot reserved for total cellular protein determination. BKV DNA copies/ml over time after administration of MPA are presented using a log scale. Total cellular protein (μg/ml) was determined using a Qubit fluorometer with the Quant-iT^{TM} Protein Assay Kit (Invitrogen Corp) after correction for culture media proteins.

mRNA analysis

Total RNA was isolated from the cellular pellet using Qiagen RNeasy® Mini Kit (Qiagen Inc., Mississauga, ON, Canada) and reverse-transcribed using a QuantiTect Reverse Transcription kit (Qiagen) according to the manufacturer's instructions. cDNA products (4 μl) were amplified by qPCR using gene-specific primers (0.5 μM) and the QuantiTect SYBR Green PCR kit (Qiagen) in a total volume of 20 μl using a LightCycler 2.0 thermocycler (Roche Diagnostics, Laval, QC, Canada). The primers used, and their sources, are shown in Table 1. Amplification consisted of a 15-min hot start (95 °C) followed by 55 cycles of denaturation (94 °C, 15 s), annealing (50 °C, 20 s), and elongation (72 °C, 20 s). Melting curves followed by separation of PCR products on a 3.0%, 1× TAE

Table 1. Primers and sources (purchased from Integrated DNA Technologies, Inc., Coralville, IA, USA).

Gene	GenBank accession no.	PCR primer sequence (5′–3′)	PCR product size (bp)
RPII	NM_000937	Fw: cgcttaagccttccaacaag Rv: gaggacgaccttgctgtctc	215
αSMA	NM_001613	Fw: ttcaatgtcccagccatgta Rv: gaaggaatagccacgctcag	222
TGF-β	NM_000660	Fw: gggactatccacctgcaaga Rv: cctccttggcgtagtagtcg	239
Col 1A1	NM_000088	Fw: acgtcctggtgaagttggtc Rv: accagggaagcctctctctc	172
HSP47	NM_001235	Fw: ccctgaaagtcccagatcaa Rv: gggagaggttgggatagagc	159

Fw, forward primer; Rv, reverse primer.

agarose gel were performed to ensure the formation of a single product at the appropriate size. Relative gene expression, normalized to the reference gene RNA polymerase II (RPII), was calculated using the $-2\Delta\Delta CT$ method [29]. RPII was used as the housekeeping gene because its expression is constant in our cell system during chronic culture and it is not influenced by BK virus infection (data not shown). To demonstrate the relative abundance of mRNA, data for MPA-exposed Vero E6 cells with or without BK virus infection are expressed relative to the corresponding levels of RPII mRNA at 1, 7, 14, 21, and 28 days postinfection.

Statistical analysis

Analysis of variance was performed by paired *t*-test, with *P*-values < 0.05 regarded as significant.

Results

Early BKV infectivity, as measured by BKV DNA copies/ml in the cellular fraction and supernatant up to 4 h postinfection, was unaffected by administration of MPA (Fig. 1), even when the dose was increased to a maximum of 16 μg/ml (Fig. 1 insert). Pre-exposure of cells to MPA (1, 2, 4, 8, 16 μg/ml) did not alter SN or cellular BKV DNA copies/ml in comparison to MPA added after BKV infection when assessed at 2 h postinfection (data not shown).

Vero E6 cells incubated with VJ isolate from urine reached a maximum of 1.E + 08 BKV DNA copies/ml 6 weeks postinfection (Fig. 2). When these cells were treated with MPA (1, 2, 4, 8, and 16 μg/ml), the increase in measured supernatant BKV DNA copies/ml over the first 7 weeks after infection was effectively suppressed in a manner that was not dose-dependent within this dose range.

Mycophenolic acid also demonstrated a significant inhibitory effect on BKV infection when Vero E6 cells were infected with a NCCR-rearranged VJ isolate (Fig. 3). However, the observed reduction in BKV DNA copies/ml with high-level infection was dose-dependent and varied from a 10^1- to a 10^4-fold reduction. Lower doses of MPA (1 or 2 μg/ml) showed no or little effect when compared with control, while higher doses (8 or 16 μg/ml) achieved significant inhibition.

Inhibition of the primary infection peak by MPA 8 μg/ml was also observed with other patient isolates of BKV (Fig. 4). When cultures were repeated using the JB and LH isolates, administration of MPA 8 μg/ml suppressed the supernatant levels of BKV DNA in each case, and cellular BKV DNA copies/ml after 8 weeks of culture. The greatest degree of inhibition occurred with the JB isolate, but in each case the suppressive effect of MPA 8 μg/ml was significant in relation to controls.

Uninfected cells exposed to MPA had significantly reduced total cellular protein (50.90 ± 10.18 μg/ml) when compared with control or infected cells (109.32 ± 73.41 μg/ml, $P < 0.0005$), consistent with an anti-metabolite action without an increase in CPE. At the end of 7-, 14-, 21-, and 28-day cultures, mRNA expression (relative to RPII) of collagen 1A1 (Col 1A1), heat shock protein 47 (HSP47), alpha smooth muscle actin (αSMA), and transforming growth factor-β (TGF-β) in Vero cells infected with NCCR-rearranged VJ isolate and/or treated with MPA 8 μg/ml were compared with mRNA expression in control Vero cells on similar days (Table 2). BKV infection was associated with transient significant

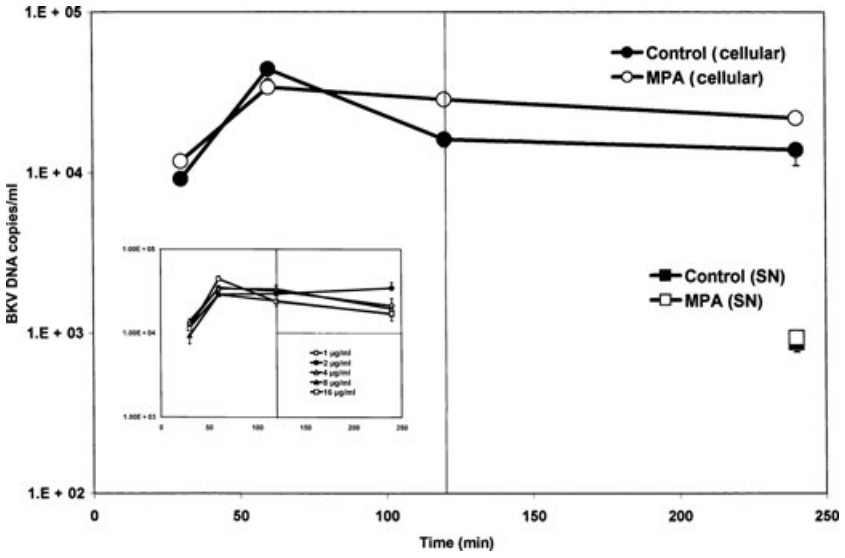

Figure 1 Early infectivity. Effect of MPA on BKV infectivity (VJ isolate) in Vero E6 cells up to 4 h postinfection. Average BKV DNA copies/ml are depicted with MPA at a dose of 1, 2, 4, 8 or 16 μg/ml versus controls in the cellular fraction and the supernatant (SN). The insert depicts BKV DNA copies/ml in the cellular fraction according to individual MPA concentration.

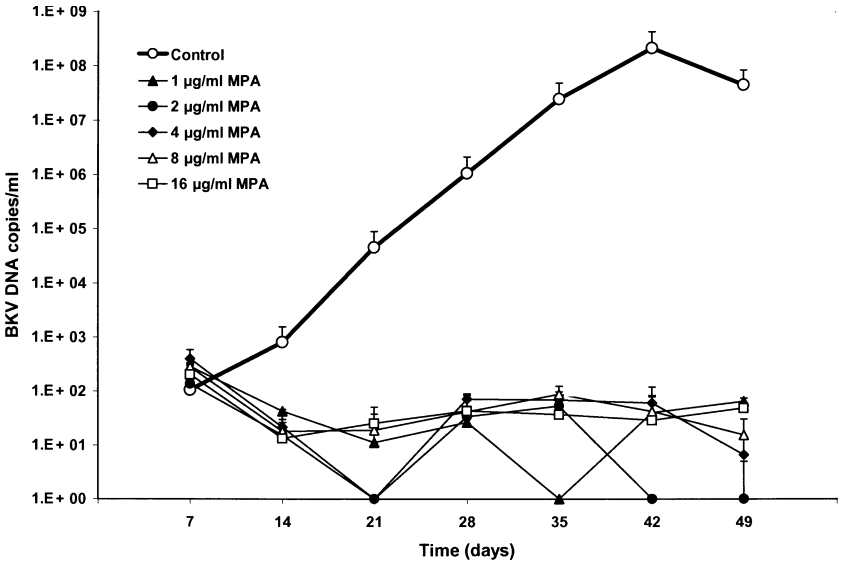

Figure 2 Primary infection. Effect of MPA versus control on the primary BKV infection peak (VJ isolate) versus control in Vero E6 cells over the first 7 weeks postinfection, according to MPA dose.

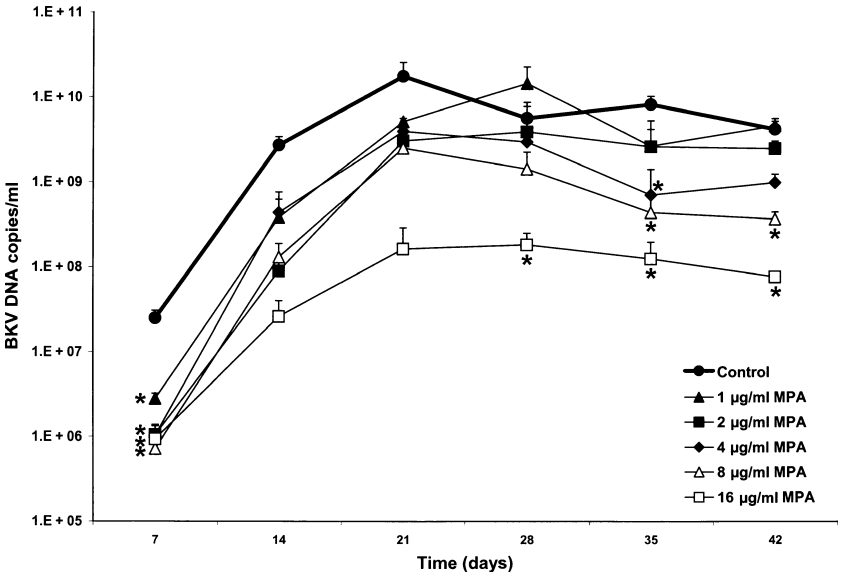

Figure 3 High-level infection. Effect of MPA versus control on the primary BKV infection peak (VJ isolate) versus control in Vero E6 cells with high-level infection ($>10^6$ copies/ml) over the first 6 weeks postinfection, according to MPA dose. $^*P < 0.05$.

reduction of Col 1A1 on day 7, which was not observed on days 14, 21, and 28 or when combined with MPA. Reduction of αSMA mRNA was observed in the BKV + MPA group on day 7 but was not apparent on any other day or in any other treatment group. No significant alteration of HSP47 or TGF-β mRNA expression was noted in any treatment grouping or time window evaluated in this chronic culture system.

Discussion

This is the first *in vitro* study of the effect of MPA on BKV replication in a cell system of kidney cell origin. Our findings demonstrate that MPA effectively prevents the primary BKV infection peak in the dose range 1–16 µg/ml, while in cells with high levels of primary BKV infection (10^6 copies/ml), doses > 8 µg/ml showed a suppressive effect. MPA administration was also observed to inhibit the ability of patient isolates of BKV to develop NCCR-rearrangements in chronic infection within the same dose range. Moreover, the anti-replicative effect of MPA was not isolate-specific, as inhibition of the primary infection peak was reproducible in various patient isolates of BKV (VJ, LH, and JB).

Mycophenolic acid administration did not affect BKV DNA copies/ml during the early phase (i.e. the first 4 h) after infection, either in the cellular fraction or in the supernatant. BKV primarily enters cells via caveolae

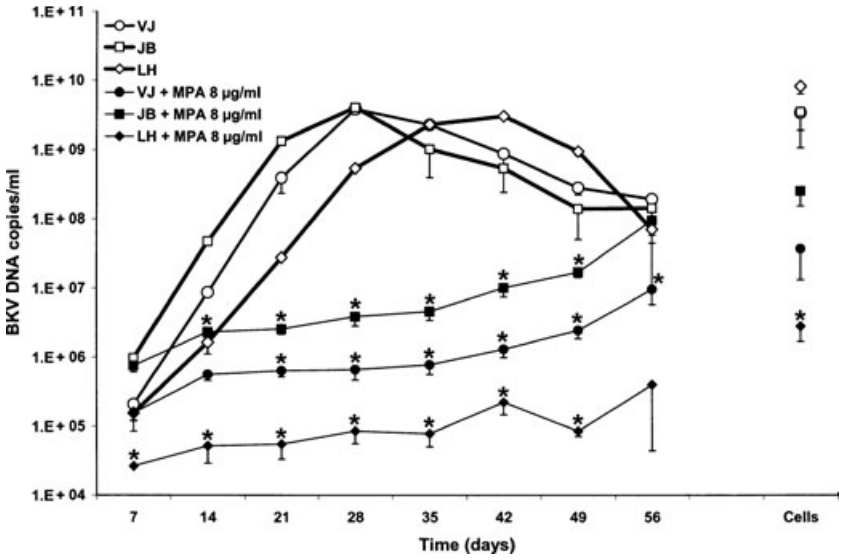

Figure 4 Effect of MPA 8 μg/ml on the primary BKV infection peak in Vero E6 cells over the first 7 weeks postinfection, according to BKV isolate (VJ, JB or LH). *$P < 0.05$.

Table 2. Changes in gene expression relative to RPII at the same time points.

	Change in gene expression				SE				*P*-value (relative to control)			
	Day 7	Day 14	Day 21	Day 28	Day 7	Day 14	Day 21	Day 28	Day 7	Day 14	Day 21	Day 28
Collagen												
Control	1.28	1.92	1.40	8.34	0.08	0.62	0.48	2.85				
BKV	**0.64**	1.78	0.22	1.34	0.18	0.52	0.07	0.45	**0.049**	0.870	0.090	0.089
MPA	1.50	0.65	0.22	0.71	0.70	0.29	0.05	0.43	0.788	0.133	0.092	0.073
BKV + MPA	1.44	0.30	0.10	2.09	0.12	0.12	0.03	0.86	0.353	0.078	0.074	0.112
HSP												
Control	1.06	2.48	0.10	2.22	0.24	0.88	0.02	0.94				
BKV	0.71	3.13	0.09	3.57	0.12	0.82	0.04	1.21	0.281	0.605	0.806	0.411
MPA	1.29	1.78	0.09	0.59	0.67	0.41	0.03	0.38	0.776	0.508	0.834	0.185
BKV + MPA	1.86	0.72	0.04	1.04	0.36	0.39	0.01	0.36	0.149	0.138	0.026	0.306
αSMA												
Control	2.63	1.16	0.20	0.94	0.00	0.47	0.09	0.26				
BKV	1.89	1.34	0.24	8.05	1.43	0.46	0.18	3.77	0.656	0.790	0.850	0.117
MPA	1.25	1.48	0.26	1.36	0.68	0.48	0.12	0.94	0.180	0.655	0.665	0.703
BKV + MPA	**1.07**	0.15	0.04	1.16	0.24	0.06	0.01	0.80	**0.022**	0.122	0.187	0.816
TGF-β												
Control	1.37	2.01	5.29	7.31	0.75	0.49	1.99	2.03				
BKV	3.28	2.41	0.73	4.02	1.20	0.58	0.32	1.20	0.323	0.619	0.105	0.224
MPA	2.08	11.34	1.41	1.06	0.72	6.24	0.55	0.35	0.530	0.246	0.179	0.156
BKV + MPA	2.14	0.68	0.35	2.79	1.21	0.28	0.19	1.86	0.621	0.069	0.089	0.152

Bold figure denotes a significant difference from controls ($P < 0.05$).

through a caveolin-1-dependent mechanism [30,31], and is transported to the nucleus by fusing with caveosomes [32], a relatively slow process that requires approximately 4 h to complete [31]. It would appear, therefore, that MPA-induced inhibition of BKV DNA replication occurs downstream of cellular entry and intracellular transport. Although further research into a possible effect on prenuclear effects is required, it seems likely that the mechanism of action of MPA, while still unclear, may be located at the nuclear level.

Determining the clinical relevance of these findings may prove challenging. We selected MPA exposure levels that reflected the range of blood concentrations observed in kidney transplant recipients, which typically vary from 1–3 μg/ml predose to as high as 8–16 μg/ml for peak concentrations [33]. The extent to which MPA suppressed

BKV primary infection was similar to our previous observations with cidofovir (2.5–25 mg/l) in the same kidney cell system. There are anecdotal reports and case series reporting favorable outcomes following low-dose cidofovir treatment in patients with PVAN, but data are limited and mixed [34]. Leflunomide has also been shown to reduce BK viral load in both blood and urine in infected kidney transplant patients [35] but this effect may not necessarily translate to the inhibition of primary infection as demonstrated in this study for MPA in our *in vitro* experiments. No prospective trial has examined the influence of MPA therapy on development of BK viruria, viremia or PVAN. Currently, the most robust data are those of Brennan *et al.* [13], who analyzed data from a randomized study of tacrolimus versus CsA in renal transplant patients and observed that MMF therapy was not independently associated with risk of VK viruria or viremia. Little information can be gained by evaluating the impact of MMF dose reduction or discontinuation following diagnosis of PVAN, as the dose of calcineurin inhibitor is usually decreased simultaneously, often with concurrent introduction of cidofovir or leflunomide [9], such that the effect of MMF dose changes on BKV infection levels cannot be determined reliably. Based on our results, however, it is conceivable that MPA dose maintenance or even increased exposure could be beneficial in patients who develop BK viremia following renal transplantation, and this possibility should be investigated as a potential therapeutic strategy.

We have previously reported direct cellular effects of other immunosuppressive agents on viral BK viral replication [28,36]. BKV has been shown to have a steroid-responsive element [37] and steroids are often used to treat rejection, which commonly correlates with PVAN. Cyclosporine suppresses early infection and NCCR-rearrangements in chronic culture in a dose-dependent manner, whereas tacrolimus does not protect Vero cells from NCCR-rearranged at doses of up to 32 μg/ml and may indeed increase the number of BKV DNA copies/ml during primary infection [36]. MPA, however, is the only immunosuppressive agent examined to date which appears to inhibit high-level BKV infection.

In conclusion, MPA inhibits primary, high-level and chronic BKV infection in this kidney cell system, an effect that was reproducible with different isolates of BK virus. At high viral loads ($>10^6$ copies/ml), this effect was dose-dependent with MPA doses >8 μg/ml remaining effective. These findings require replication elsewhere, and the mechanism through which BKV DNA replication is restricted by MPA needs to be elucidated. The potential clinical implications of a direct anti-replicative effect of MPA on BK polyoma virus are interesting; however, the same merit investigation. Our *in vitro* data suggests that MPA might have a protective effect in BKV-infected renal tubular cells, with both an anti-viral and an anti-fibrotic response. Hence, the current clinical strategy of reducing all classes of immunosuppressants in patients with BK viremia and/or BKV nephropathy may not be the optimal strategy. Our *in vitro* data suggest that it may be appropriate to compare the effect of maintaining calcineurin inhibitor exposure while reducing MPA dose versus decreasing calcineurin inhibitor exposure without altering MPA levels in kidney transplant recipients with early BKV nephropathy or with BK viremia.

Authorship

PDA: designed research/study, secured resources, and wrote paper. PAO'R: performed research/study and contributed to editing of paper. JFSC: contributed to design of research/study and helped secure resources.

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