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Characterization of BK viral responses to the dual-PI3K/MTOR inhibitor dactolisib (NVP BEZ-235) in a renal cell culture model

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Boston University
CHARACTERIZATION OF BK VIRAL RESPONSES TO THE DUAL-PI3K/mTOR INHIBITOR DACTOLISIB (NVP BEZ-235) IN A RENAL CELL CULTURE MODEL

by

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B.A., Colby College, 2012

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DEDICATION

I would like to dedicate this thesis to my family, friends, girlfriend, and everyone else in my life who has supported and encouraged me in pursuing my passions, be they academic, extracurricular, artistic, or interpersonal.
ACKNOWLEDGMENTS

First of all, I would like to acknowledge the support and patience of Sabrina, Matt and Tom, and Dr. Offner, all of whom were incredibly helpful in making this project a reality. Thank you for your careful explanations of how to do things in lab, weekly discussion of results and experimental design, putting up with my endless questions and requests, and reading drafts of this thesis. Tom, thank you for providing plasmid pBR-322, as well as growing up stocks of BK virus itself. Xin, thank you for discussing Western blot trouble-shooting techniques and the kind gift of SV40 776 supernatant for some of my experiments. Sabrina, thank you for being an excellent mentor and encouraging me throughout the entirety of this process.

Furthermore I would just like to acknowledge everyone else who has helped me make it to this point: Frank Fekete, Lance Lanoy, Mailiani Bailey, Bruce Reuger, Cate Ashton, Herb Wilson, Ethan Kohn, Rob Janett, Tariq Amhad, Andrew Buckman, Naomi Heindel, and David Smith.
CHARACTERIZATION OF BK VIRAL RESPONSES TO THE DUAL-PI3K/mTOR INHIBITOR DACTOLISIB (NVP BEZ-235) IN A RENAL CELL CULTURE MODEL

GABRIEL BENJAMIN WARACH LERNER

ABSTRACT

BK virus (BKV) is a ubiquitous polyomavirus known to asymptptomatically reside in the renal tissues of up to 90% of the human population. BK virions reactivate during periods of intense immunosuppression and can cause disease in renal transplant recipients, such as BKV-associated nephropathy (BKVAN). BKVAN can lead to loss of the transplanted renal grafts. For this reason, the study of BKV biology is of importance to the transplant community. Previous studies have shown that BKV upregulates the pro-growth mTOR pathway in host cells, thereby increasing BKV replicative efficiency. Downstream effectors of the mTOR pathway, particularly p70S6 kinase, control the basal rate of protein translation, in part through regulation of ribosomal biogenesis. It was hypothesized that viral upregulation of the mTOR pathway is beneficial for viral replication due to an increase in the number of ribosomes available to translate viral proteins. Therefore, inhibition of the mTOR pathway could reduce viral replication. This study investigated whether host cell mTOR inhibition could reduce BK viral replication in an in vitro model. We utilized the dual PI3K/mTOR inhibitor NVP BEZ-235 (Novartis Pharmaceuticals), which potently downregulates expression of both upstream (PI3K) and central (mTOR) effectors of the mTOR pathway.

Immortalized renal epithelial cells were exposed to varying concentrations of BEZ-235 for a period of 48 hours, infected with BK virus for three hours, and allowed to...
grow for a further 48 hours. Cell populations were then assayed via quantitative PCR (qPCR), Western blotting and fluorescent immunohistochemical staining to determine the effect of BEZ-235 on BK viral replication.

Western blot experiments confirmed the effectiveness of BEZ-235’s inhibition of the mTOR pathway in a renal epithelial cell culture model, as well as downregulation of the mTOR pathway during BK viral infection. Western blotting for the key BK replicative protein Large T antigen, showed a dose-dependent decrease in expression, with increasing concentrations of BEZ-235. Fluorescent immunohistochemical staining showed a dose-dependent decrease in expression of Large T antigen staining in host cell nuclei. qPCR results were inconclusive, in that no clear pattern in the number of BKV genomes per cell population could be observed across the range of BEZ-235 concentrations tested.

While results from our study indicate that BEZ-235 can reduce BKV replication in vitro, further in vitro experimentation, including repetition of approaches already carried out as well as novel approaches, will be needed to definitively confirm inhibition of the mTOR pathway as a viable antiviral strategy.
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<table>
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<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>Akt</td>
<td>See PKB</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>BKV</td>
<td>BK virus</td>
</tr>
<tr>
<td>BKVAN</td>
<td>BK Virus Associated Nephropathy</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-Dependent Kinase</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal Fluid</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>ERAD</td>
<td>Endoplasmic Reticulum Associated Degradation pathway</td>
</tr>
<tr>
<td>GVHD</td>
<td>Graft versus Host Disease</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency virus</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>JCV-GCN</td>
<td>JC Virus Granular Cell Neuronopathy</td>
</tr>
<tr>
<td>JCV-E</td>
<td>JC Virus Encephalopathy</td>
</tr>
<tr>
<td>JCV</td>
<td>JC Virus</td>
</tr>
<tr>
<td>KIPyV</td>
<td>Karolinska Institute Polyomavirus</td>
</tr>
<tr>
<td>LgT</td>
<td>Large Tumor antigen</td>
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<tr>
<td>MCPyV</td>
<td>Merkel Cell Polyomavirus</td>
</tr>
<tr>
<td>MWPyV</td>
<td>Malawi Polyomavirus</td>
</tr>
<tr>
<td>NCRR</td>
<td>Non-coding Regulatory Region</td>
</tr>
<tr>
<td>NGS</td>
<td>Normal Goat Serum</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian Target of Rapamycin</td>
</tr>
<tr>
<td>mTORC1,2</td>
<td>mammalian Target of Rapamycin complex 1, 2</td>
</tr>
<tr>
<td>PBMCs</td>
<td>Peripheral Blood Mononuclear Cells</td>
</tr>
<tr>
<td>PBS (d)</td>
<td>[Dulbecco’s] Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PH</td>
<td>Plextrin Homology (domain)</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>PML</td>
<td>Progressive Multifocal Leukoencephalopathy</td>
</tr>
<tr>
<td>PRAS40</td>
<td>proline-rich Akt Substrate of 40 kilodaltons</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative (‘real-time’) Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SmT</td>
<td>Small Tumor antigen</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian Virus 40; Simian Vacuolating virus</td>
</tr>
<tr>
<td>TBS (-T)</td>
<td>Tris-Buffered Saline (with .05% Tween)</td>
</tr>
<tr>
<td>TSC2</td>
<td>Tuberous Sclerosis Complex 2</td>
</tr>
<tr>
<td>VP1</td>
<td>Major Viral Protein 1</td>
</tr>
<tr>
<td>VP2, VP3</td>
<td>Minor Viral Proteins 2 and 3</td>
</tr>
<tr>
<td>WUPyV</td>
<td>Washington University Polyomavirus</td>
</tr>
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INTRODUCTION

Motivation for Study of the Polyomaviruses: SV40, JC virus, and BK virus

Polyomaviruses are a diverse group of species-specific, non-enveloped, double-stranded DNA viruses that are ubiquitous in nature (1). Some members of this family are thought to latently, asymptomatically infect a large percentage of humans (Table 1):

Table 1. Seroprevalence of known human polyomaviruses in healthy adults. ‘Seroprevalence’ refers to the proportion of samples in a population that are positive for serum antibodies against species-specific viral proteins, as a result of prior immune exposure. BKV is highlighted in red. Modified from Dalianis and Hirsch, 2013 (2).

<table>
<thead>
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<th>HPyV</th>
<th>Seroprevalence in adults (%)</th>
<th>Country</th>
<th>Method</th>
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<tr>
<td>BKV</td>
<td>82–99</td>
<td>USA, Australia, Italy</td>
<td>VLP ELISA and VP1 capsomer based ELISA</td>
</tr>
<tr>
<td>JCV</td>
<td>39–81</td>
<td>USA, Australia, Italy</td>
<td>VLP ELISA and VP1 capsomer based ELISA</td>
</tr>
<tr>
<td>KIPyV</td>
<td>55–90</td>
<td>USA</td>
<td>VLP ELISA and VP1 capsomer based ELISA</td>
</tr>
<tr>
<td>WUPyV</td>
<td>69–98</td>
<td>USA</td>
<td>VP1 capsomer based ELISA Multiplex antibody binding assays</td>
</tr>
<tr>
<td>MCPyV</td>
<td>60–81</td>
<td>Italy</td>
<td>VLP ELISA Multiplex antibody binding assay</td>
</tr>
<tr>
<td>HPyV6</td>
<td>69</td>
<td>USA</td>
<td>VLP ELISA</td>
</tr>
<tr>
<td>HPyV7</td>
<td>35</td>
<td>USA</td>
<td>VLP ELISA</td>
</tr>
<tr>
<td>TSV</td>
<td>70</td>
<td>The Netherlands</td>
<td>Multiplex antibody binding assay</td>
</tr>
<tr>
<td>HPyV9</td>
<td>21–53</td>
<td>France, Germany</td>
<td>VLP ELISA VP1 recombinant protein ELISA</td>
</tr>
<tr>
<td>MWPyV/ HpyV10</td>
<td>ND*</td>
<td></td>
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* Not done.

Although most polyomaviruses are thought to be clinically harmless, several have been implicated in disease. Simian Virus 40 (SV40) was the first non-human
polyomavirus to be isolated, after having been found in contaminated rhesus monkey renal cell cultures used to produce poliovirus vaccine (3). SV40 contamination was detected in both Salk (inactivated) and Sabin (live-attenuated) vaccines: it has been estimated that over 98 million people were exposed at the time (4). For the next several decades, the question of whether SV40 is capable of causing cancerous growth of human cells emerged as one of the most controversial in all of cancer research (4). Subsequent studies investigated the epidemiologic and transformative consequences of this mass contamination event, and concluded that there was insufficient evidence to suggest that the virus was oncogenic (4). These decades of intensive study, however, have shed much light on the biology, life cycle, and virulence mechanisms of Polyomaviruses in general.

SV40 is also known as Simian Vacuolating virus, for the intracellular vacuoles that these virions induce internally in productively infected simian cells (3). Currently, strains of SV40 are used by researchers largely as a tool to create ‘immortalized’ cell lines. Pro-growth SV40-derived genes are artificially inserted into primary cell genomes, inducing them to grow indefinitely in proper culture conditions. While it has not been shown to induce disease in humans, SV40 remains the single most well-studied polyomavirus. Its attributes, viral lifecycle, and genomic structure have been used to inform the study of the biology of the more clinically relevant polyomaviruses: JC virus (JCV) and BK virus (BKV). In a very real way, the scientific lives of these latter viruses exist in the shadow of SV40, their more well-studied cousin.

JCV was discovered in 1971 by Padgett et al., and eventually implicated as the etiologic agent of the rare and often fatal brain disease, progressive multifocal
leukoencephalopathy (PML) (5). BKV and JCV resides latently in the kidneys, bone marrow, and lymph nodes of healthy persons (6). Immunosuppressed states, such as those caused by chronic HIV infection, organ transplantation or the treatment of some cancers, permit JCV to reactivate within the oligodendrocytes of the central nervous system (7). Oligodendrocytes produce myelin sheath that wraps around neurons, enabling signal conduction in the CNS. Such selective tropism may cause localized immune system activation and formation of the foci that are characteristic of PML.

PML-associated foci are small (typically 1-3 mm in diameter) distinct areas of demyelination that usually appear in the white matter of the brain (8). Lesions are necrotic in nature, and produced secondary to destruction of oligodendrocytes following lytic JCV infection (8). PML, as the name suggests, is a progressive disease – lesions that first appear as solitary entities may later become ‘clumped’ in groups and merge together over time (6). Recent studies suggest that JCV may also be capable of independently infecting the meninges covering the outer surface of the brain, as well as neurons in the granule cell layer of the cerebellum and pyramidal neurons in the cortex (9, 10). Such tropic infections cause ‘JCV-Meningitis’ (JCV-M), ‘JC Virus Granular Cell Neuronopathy’ (JCV-GCN) and ‘JC Virus Encephalopathy’ (JCV-E), respectively (9-11). Further studies are needed to elucidate the molecular mechanisms responsible for the tropism of JC virus to these distinct cell types, as well as the full implications of such tropism on the clinical presentations and syndromes observed in patients with JC virus-associated pathology.
In the past decade, the introduction of novel genetic sequencing techniques has led to the discovery of many other members of the Polyomavirus family. These include Merkel cell polyomavirus (MCPyV), which has been implicated in the pathogenesis of Merkel Cell Carcinoma, KIPyV and WUPyV (named after the institutions where they were discovered; the Karolinska Institute in Stockholm, Sweden, and Washington University in St. Louis, respectively), and the rationally named Human Polyomaviruses 6, 7, and 9, among others (2). Viral genotypic analyses have confirmed a high degree of sequence similarity between BK, JC, and SV40 viruses, implying close common ancestry as well as shared growth, infection, and virulence mechanisms (Figure 1; (12)).
Figure 1. Phylogenetic analysis of the evolutionary relationships among members of *Polyomaviridae*. Malawi Polyomavirus (MWPyV), a newly discovered polyomavirus originating in Malawi, is bolded, and the clade containing BKV, JCV, and SV40 is outlined in red. BKV, JCV, and SV40 share a high degree of sequence homology. Amino-acid based phylogenetic trees were generated using the maximum likelihood method, with 1,000 bootstrap replicates. A) represents relationships determined via VP1 sequence, B) via VP2 sequence, and C) via Large T antigen sequence. Figure modified from Siebrasse, et al., 2012 (12).

**Discovery and Characterization of BK Virus**

BKV is a double-stranded, non-enveloped, human DNA polyomavirus with an icosahedral capsid 40 – 44 nm in diameter. It has a genome of approximately 5,200 base pairs and was first isolated from virally infected cells in the urine of a renal transplant recipient (initials ‘BK’), in London in 1970 (13). These cells were examined via light and electron microscopy, and found to exhibit enlarged, activated nuclei, with high concentrations of viral particles (Figures 2, 3; (14)). Subsequent cellular culturing confirmed the presence of the virus (14).
Figure 2. Light microscopy image of urinary ‘decoy cells’ containing BKV. ‘N,’ nucleus; ‘C,’ cytoplasm; 400x magnification. Note the enlarged nucleus (greater than average nucleus to cytoplasmic ratio) and ‘ground-glass’ appearance, indicative of viral stimulation of host cell transcription and translation. Visualized with the Papanicolaou stain. Image modified from Ashan, 2006 (15).

Figure 3. Electron microscopy images of BKV-infected renal epithelial cells. Figure 1A shows BK virions isolated from the urine of patient ‘BK.’ Figure 1B shows two renal epithelial cells in a nephron (‘L’ shows the nephron luminal space), with significant viral inclusions (‘V’) found within the cell nuclei (‘N’); corner inset of this image shows clusters of BK virions. Figure 1A is 180,000x; Figure 1B is 6000x, and inset is 22,500x. Figures modified from (Gardner, et al., 1971), with permission from the publisher (16).

Since its discovery, BKV has been implicated as an important human pathogen that causes disease in immunosuppressed populations including: transplant recipients, patients undergoing chemotherapy, and those infected with Human Immunodeficiency Virus (HIV).
Primary BK Infection

BKV is virtually ubiquitous in man; some 70 – 99 % of the world’s human population is thought to be latently infected (2, 14). In fact, by tracking the evolution and mutational status of BKV strains in contemporary human populations across the globe, scientists have been able to confirm and substantiate early human migratory patterns (17). Primary infection with BKV occurs asymptomatically during infancy or early childhood, through the oral route. Although the initial acquisition of this virus is poorly understood, mechanisms of transmission are presumed to be through environmental, respiratory, urinary, or vertical (maternal to fetal) means (18, 19). The first site of invading BK virus populations may be in the tonsillar tissue, followed by trafficking to the kidneys via infection of peripheral blood mononuclear cells (PBMCs) that circulate through the secondary immune tissues of the tonsils (20, 21).

As a nephrotropic virus, BKV homes to, and establishes latency within, the epithelial cells of the kidney and genitourinary tract (22). BK viral latency is defined by quiescence; the virus persists inside the host cell without attempt to replicate itself. Latent BKV can exist in the body for the entire life of the individual, without progression to viral reactivation or disease. The BK viral genome exists in an episomal form in the nucleus of human cells, although it has been shown to incorporate itself into the host genomes of some rodent cell lines (20).
BK Virus Genome and Viral Proteins

The BK viral genome is circular, double-stranded, and approximately 5,100 base pairs in length, with six well-defined protein encoding genes and a non-coding regulatory region (Figure 4; (20)):

Figure 4. Genomic map of BK virus, Dunlop strain. Approximate genome length: 5,153 base pairs; transcribed in both directions from the origin of replication (‘ori;’ green), via a temporally-regulated mechanism (not simultaneously). The origin of replication is located within the noncoding regulatory region (NCRR; green). Large and small t antigen (in blue) are the so-called ‘early genes,’ which are transcribed and translated early in the viral life cycle. VP1, VP2, and VP3 (in red) are the ‘late
genes,’ which code for BK capsid proteins. The function of BK agnoprotein (purple) is currently under study, but the protein may be involved in nuclear localization of maturing virions, and release of viral progeny during lytic episodes (41). Figure modified from Cubitt, et al., 2013 (20).

Like all polyomaviruses, the BK viral genome contains coding strand DNA on both strands, rather than having a single continuous coding and non-coding strand (1). The six genes include Large Tumor antigen (LgT), Small Tumor antigen (SmT), major viral capsid protein 1 (VP1) and minor viral capsid proteins 2 and 3 (VP2 and VP3), as well as the BK Agnoprotein (Figure 5). The early genes are large and small T antigen. Large T, in particular, plays a major regulatory role and is required for viral replication and productive infection (14). One of Large T antigen’s major functions is to bind to and inactivate the tumor suppressor proteins Rb and p53, thereby removing a key block on the host cell’s entry into the cell cycle (42).

Late gene products include VP1, VP2, VP3, and BK Agnoprotein. VP1 comprises the majority of the BKV capsid surface area; for every VP2 or VP3 capsid subunit present on the capsid surface, there are five VP1 subunits (14). VP2 and VP3 transcripts are generated from the differential post-transcriptional processing of a single mRNA transcript (14). The topology of the BK capsid is such that VP2/3 expression can only be detected after partial proteolytic degradation and viral uncoating in the endoplasmic reticulum (Figure 5; (25)). Thus, VP2/3 expression can be used as a marker of BKV ER localization (25).
Figure 5. Diagram showing topographic relationship between VP1 (green) and VP2 (purple) BKV capsid proteins along a single axis. ‘C’ and ‘N’ denote carboxy- and amino-termini of the VP2 primary amino acid protein sequence. Note that if viewed in three dimensions, three other VP1 subunits would surround and encapsulate the VP2 subunit, thus effectively ‘sheltering’ the latter from the extracellular space. Figure modified from Kahlili and Stoner, 2001 (8).

Certain BK genes, such as Large T antigen, are transcribed and translated early in the BKV lifecycle, while others, such as VP1, appear later in the viral lifecycle. This distinction can be taken advantage of experimentally. Large T antigen can be interpreted as a marker solely of viral entry, rather than productive infection, whereas presence of VP1 DNA can be seen as a marker of productive (i.e. successful) infection, where BK capsid proteins are being constructed inside the host cell, in preparation for release into the extracellular environment.
The exact function of the final protein of the BK genome, BK Agnoprotein, remains contested. It is thought that BK Agnoprotein assists in BK virion assembly and release, by acting as a nuclear localization signal for VP1 during capsid formation, and as a viral ionic channel in host cell membranes (41, 43).

The non-coding regulatory region (NCRR) contains the origin of replication as well as several transcription factor binding sites, and is often hyper-mutated (rearranged) in productively-infecting BK strains (14). Wild-type BK virus possesses a non-rearranged form of the NCRR; rearranged BK strains are often found in the epithelial cells of renal biopsies of patients with BKVAN (14). It has been hypothesized that rearrangements must occur in order for the virus to become capable of productive infection (20).

Transcription and translation of all BKV genomic proteins are critically facilitated the expression of Large T antigen. Factors affecting the expression of Large T antigen will therefore secondarily affect levels of all BKV proteins, as well as the rate and efficiency of BK viral replication overall. Such factors include BKV’s ability to productively infect the host cell (and deliver its genome to the nucleus; discussed below); the degree to which BKV can establish latency and evade host immune surveillance mechanisms; and/or the availability of host cell replicative machinery (i.e. competition with host cell molecules for access to DNA polymerases or ribosomal components). This third factor is critical in the rationale for using an mTOR pathway inhibitor as an antiviral therapy.
Cellular Basis of BKV Attachment and Infection

Many steps in BK viral infection, from attachment and virion trafficking, to nuclear entry and genome replication, are cell-type specific. A consensus has been reached, however, regarding certain intracellular events shared across cell types. BKV preferentially attaches to renal epithelial and urothelial cells, via ganglioside motifs present on its capsid, and the host cell ganglioside receptors GD1a and GT (Figure 6 below, shows a proposed model of BKV infection; (23)).

BKV internalization is accomplished by a caveolin-mediated endocytotic mechanism – one similar to, although independent of, clathrin-mediated uptake. Caveolae are detergent-resistant, cholesterol rich membrane invaginations that bud internally into the cytoplasm after BK viral binding to extracellular receptors. Caveolae are stabilized by caveolin-1 expression (24).

Once internalized, BKV hijacks a number of host cell pathways and processes to facilitate its transport into the host cell nucleus (Figure 6). BKV-containing vesicles become acidified, causing partial viral decoating and breakdown of disulfide bonds, and migrate in a retrograde fashion along microtubules towards the trans-face of the Golgi apparatus (25). BK virions subsequently move towards the cis-face of the Golgi apparatus, and bud into vesicles that migrate to the rough ER (rER). Arrival to the rER has been postulated to be eight hours post viral infection, although the specifics of how
BKV controls the signaling and temporal nature of these retrograde trafficking events have yet to be elucidated (25, 26). Recent evidence indicates that BKV then enters the cytosol from the rER by hijacking the endoplasmic reticulum associated degradation (ERAD) pathway (27). The ERAD is a large group of ER-associated trans-ER-membrane proteins that recognize and remove misfolded or abnormal proteins as they arise over the course of modification in the ER (27). Proteins targeted by ERAD machinery are poly-ubiquitinated as they are shunted into the cytosol, triggering their localization to proteasomes, where protein disassembly occurs (27). ERAD has also been implicated in the regulation of overall levels of certain proteins in the cell, such as HMG CoA reductase, the rate-limiting enzyme involved in cholesterol synthesis. During times of adequate or excessive cholesterol, ERAD machinery is selectively up-regulated to target HMG-CoA reductase (27). When seen in this light, the ERAD pathway could be seen as a form of post-translational regulation.
Figure 6. Model of the proposed major steps in BK viral infection of renal epithelial cells. Binding of BKV to ganglioside receptors on the host cell surface (A) triggers caveolin-mediated endocytosis (B). The BK-containing endosome subsequently merges with an acidic endosome (not shown), which decreases pH and begins the viral uncoating process (C). Recent studies have shown that the virion-containing endosome then directly merges with the endoplasmic reticulum (E) and further viral uncoating occurs. BK virions hijack Endoplasmic Reticulum Associated Degradation (ERAD) transmembrane proteins (such as Derlin-1) present in the ER to facilitate their transport into the cytosol (F). The low-Ca\textsuperscript{2+} environment in the cytosol further facilitates viral uncoating and exposure of the BK genome (26). The genome is then transported via an unknown mechanism into the host cell nucleus. Figure modified from Jiang, et al., 2009 (26).
BK Viral Reactivation

Under certain circumstances, BK viral reactivation may occur, as previously latent BKV switches to a lytic phase inside the host cell. Once reactivation is triggered, the virus begins to replicate its genome and use host cell machinery to transcribe and translate viral proteins, in preparation for cell lysis and the release of viral progeny. This reactivation can occur in a variety of situations and tissues, and lead to several uniquely defined disease states (Figure 7).

Figure 7. Common predisposing factors of BK viral reactivation, and potential pathologies associated with such reactivation. Importantly,
low levels of BK reactivation and viruria can persist without progression to viremia or disease. Iatrogenic causes of immunosuppression include immunosuppressive medications given during cancer treatment (chemotherapy, radiation therapy), as well as pre- and post-organ transplantation immunosuppression therapy. BKVAN: BK virus associated nephropathy.

Although the molecular triggers for reactivation are poorly understood, physiologic stress of host cells, moderate or severe immunosuppression, corticosteroid use, and physical trauma may all promote viral reactivation (28). In general, these triggers collectively activate pro-growth or cell cycle regulatory components which render the host cell permissive for viral replication (28). One such pro-growth intracellular signaling circuit, the “phosphatidylinositol-3-kinase/Akt⁠¹/mammalian target of Rapamycin” (PI3K/Akt/mTOR) pathway, has been shown to be activated by BKV, and is currently under study as a target for BKV antiviral treatments (22).

**BK Virus Associated Nephropathy**

BKV reactivation is a known risk factor for a common BK-induced disease, BK-virus-associated nephropathy (BKVAN; (14)). BKVAN is the direct result of BK viral reactivation and replication in the renal urinary tract, and is similar histologically to nephropathy caused by other viruses such as CMV or JCV (29). BKVAN is defined as a persistent (>3 weeks) period of BK viruria, a plasma PCR viral load of greater than 10,000 genomes/mL, as well as histological evidence of productive BKV infection in the

¹ Akt is also referred to as Protein Kinase B (PKB).
kidney (29). Renal biopsy of focal BK-positive lesions remains the diagnostic gold standard for this disease (29, 30).

The majority of the structural and cellular damage to the kidney in BKVAN may be due to immune system invasion and destruction of the tissue, rather than directly due to the virus’ lytic reactivation (13). 1 – 10% of renal transplant recipients experience some degree of BKVAN, which has become recognized as a leading cause of graft failure (31). BKVAN diagnosis is made on average 44 weeks post kidney transplantation, with a mode of 24 weeks (32). The most common complication related to BKV reactivation is uretal stenosis in bone marrow transplant patients, which occurs (from all causes) in 10-25% of bone marrow transplant recipients (1).

Overall immunosuppression is most severe in the acute post-transplant phase; thus it is during this period that renal transplant patients are most likely to develop BKVAN. Immunosuppressive burden is the single most important predictor of BKVAN prognosis (1). Other prognostic factors include measures of the BK serum viral load, severity of cytopathic cellular changes in infected renal tubule cells, and promptness of clinical diagnosis (1). The severity of BKVAN in the renal transplant setting, as well as other diseases associated with BK virus, warrant further research into strategies to reduce BK viral replication.
Difficulties in BKVAN Diagnosis

The nature of BKVAN, however, makes a straightforward clinical diagnosis difficult. There are diagnostic and technological realities that often reduce the level of certainty with which the clinician can draw conclusions about the presence or absence of BKV-associated renal pathology (14). As an example, since BKVAN is a focal disease, a negative biopsy result does not necessarily rule out its presence (31). Thus, clinicians have had to resort to making predictions based on incomplete information, relying on trends in systemic levels of the virus over time.

Detection of histopathological urinary decoy cells that contain BK virions may also represent a confounding variable for clinicians attempting to diagnose BKVAN (see Figure 2, page 6). Such decoy cells are shed during active replication and BKVAN, but are also shed asymptomatically during periods of normal health, by up to 54% of individuals (33, 34). Furthermore, histologically identical decoy cells are also produced during active JCV and adenovirus infections (35, 36).

An additional difficulty facing clinicians is a wide differential diagnosis in patients who may have BKVAN. Potential pathologies producing a similar clinical picture include acute kidney rejection, GVHD, or other viral kidney infections that result in a similar presentation (13). Two major factors that predispose individuals towards the development of BKVAN are (1) the relative efficacy of BK viral replication, and (2) the failure of the host immune surveillance mechanisms designed to keep viral replication in check: i.e. BK-specific CD-8+ T cell responses. Assays currently developed in
immunology laboratories may in the future provide clinicians with tools to monitor the status of these markers of BK disease progression. These tools include viral challenge, immunophenotyping, and cytokine release assays, which characterize host immunological fitness and evaluate virus-specific immune responses (13).

**Monitoring BK Viral Load: the Quantitative Polymerase Chain Reaction Assay**

The BK ‘viral load’ is an estimation of the number of viral genomes present in a body compartment. The status of BK replication in at-risk patients can be inferred from the viral load in both the blood and urine, using quantitative PCR (qPCR) assays. qPCR is a common molecular biology technique used to simultaneously amplify and quantify the original number of copies of genetic material in a sample (37). This is accomplished by adding several components to samples containing ‘template DNA,’ which is the specific section of DNA (mammalian, viral, or otherwise) being artificially amplified in the laboratory. These components include: (1) Taq Polymerase, a heat-stable DNA polymerase originally isolated from the bacterial thermophile species *Thermus aquaticus*, (2) a Fluorescent ‘reporter’ dye attached to a probe, which is designed to dissociate from the probe/DNA polymerase complex when replication occurs, and reattach after the process has finished, (3) single nucleosides used to synthesize new DNA strands, and (4) ions and other molecules in a buffered solution (37). The sample is then placed in a PCR machine and heated at various temperatures to facilitate the creation of ‘amplification cycles’ (Figure 8). This assay is also known as ‘real-time’ PCR, because copy number is
assessed (florescence is measured) at the end of each and every amplification cycle, in ‘real time.’

Figure 8. Model of BKV DNA amplification process during the quantitative (‘real-time’) Fluorescent Polymerase Chain Reaction assay. Amplification cycles consist of repeated (x40), controlled heating of the sample to specific temperatures for specific lengths of time, in discrete denaturation (1), annealing (2), and elongation (3) steps. During the denaturation step (1), the sample is heated to 95°C. At this temperature, double-stranded DNA dissociates into single strands, which can then be accessed by the forward and reverse RNA primers (which facilitate polymerase binding), a Fluorescent RNA probe and Taq
Polymerase. Annealing (2) of the RNA primer upstream of the target of interest occurs next, in this case a specific region of the BK VP1 gene, at the optimal temperature of 60°C. A final elongation step (3) occurs simultaneously with annealing, at 60°C (this is not always the case, but happens to be for VP1 amplification specifically). During this step, Taq Polymerase molecules, which are present in excess in the sample, bind to and replicate all target DNA in the sample.

Repetition of these steps occurs sequentially for 40 cycles, and results in exponential amplification of the target sequence specifically. The RNA probe is responsible for producing the Fluorescent signal that is detected by the PCR machine during each amplification cycle. Most RNA probes used in qualitative PCR are designed to contain two Fluorescent dyes, a ‘quencher’ and a ‘reporter’ (38). These two dyes remain in close proximity to one another on the probe, in a ‘quenched’ (non-Fluorescent) 3D conformation. However, when the probe is bound to a section of DNA, and Taq polymerase moves past it, the conformation of the probe is changed in such a way as to release the ‘quencher’s’ inhibition of the reporter dye. No longer inhibited, the reporter dye then fluoresces at a specific wavelength which can be measured by a detector within the PCR machine.

Importantly, the ‘quencher’ reattaches to the probe after each amplification cycle, resuming its inhibition of ‘reporter’ fluorescence (38). In this way, fluorescence is emitted during each cycle – although the PCR machine is not sensitive enough to measure fluorescence emitted during the earlier cycles (1-20), it is able to use fluorescence data from later cycles to extrapolate ‘backwards’ to determine the original quantity of target DNA in the sample. This ability makes qPCR an attractive tool for measuring viral loads (i.e unamplified DNA) in patient blood, urine, cerebrospinal fluid (CSF), or respiratory sputum. Image modified from: http://serc.carleton.edu/microbelife/research_methods/genomics/pcr.html.

Add in 2 min incubation at 50C; this is to ‘clean the sample up’ by digesting any residual components that may be present; prior to actual amplification. Uracil instead of tymidine? UDG?

Because of the risk of BK viral reactivation during organ transplantation, it is now standard procedure for renal transplant teams to monitor BK viral status in the blood and urine at regular intervals, both pre- and post-transplant, using qPCR. BKV generally
appears in the urine (viruria, which can be asymptomatic) before it can be appreciably measured in the bloodstream (viremia). One study showed that BK virions are shed periodically in the urine by up to 40% of immunocompetent adults, implying that immunosuppression is not required for viral reactivation (13, 39).

**Treatment of BKV-Induced Disease**

To date, there are no BKV-specific antiviral agents approved by the FDA for clinical use. Current treatment approaches for BKVAN and other BK-induced diseases center around early and consistent monitoring of BK viral load, both pre- and post-transplant, and adjustment of the patient’s immunosuppressive regimen, if needed. Although some antiviral medications (e.g., cidovir, leflunomide, quinolones, or intravenous immunoglobulin G) are commonly prescribed by physicians, their use has not been validated or standardized, and they have had limited clinical effectiveness (1). If BK-induced disease is suspected (i.e., if a decline in kidney function accompanied by a plasma BK viral load of ≥ 10,000 genomes/mL), a prompt reduction of the systemic immunosuppressive medications used to help prevent organ rejection is generally indicated (30).

The rationale is that by lifting iatrogenic immune suppression, the host immune system will more efficiently eliminate BK-infected cells (22). As might be expected, this approach can easily result in harm to the patient, as a reduction of immunosuppression
increases the likelihood that recipient immune system cells will target and attack the donor organ.

Despite the risk of organ rejection, the alternate consequence, that of unchecked BKVAN, is the more immediate concern when deciding how to manage immunosuppression in the face of presumed BK viral reactivation. BK-associated disease in the setting of kidney transplantation is a significant cause of kidney graft rejection (29). Further randomized clinical trials are needed to define the best strategy for immunosuppression reduction in the setting of polyomavirus-mediated disease. In addition, better antiviral agents are required, given the current poor outcome of grafts after development of BK nephropathy (40). The development of targeted anti-BK therapies stands to provide enormous benefit to transplant medicine.

Overview of the PI3K/Akt/mTOR Pathway

The pro-growth “phosphatidylinositol-3-kinase (PI3K)/Akt/mammalian target of Rapamycin” (mTOR) pathway is a key intracellular signaling pathway in host cells. The mTOR pathway is critically and centrally involved in regulation of multiple ubiquitous intracellular cycles, including cell proliferation, apoptosis, survival, metabolic activities, and regulation of protein translation (44, 45). There are several regulatory and feedback mechanisms in place to prevent excessive or reduced activity of this pathway (46, 47). External inputs to the mTOR system include nutrient availability, hypoxia, and growth factor binding the host cell surface (46).
Phosphoinositide-3-Kinase

Phosphoinositide-3-Kinases (PI3K) are a diverse class of protein kinases that regulate many key cellular functions via phosphorylation (48). One class of PI3Ks, Class I, represents the most upstream regulatory component of the mTOR pathway. Class I PI3Ks are comprised of a p110 catalytic subunit, and a p85 regulatory subunit (49). Both of these subunits represent important substrates for targeted pharmacological inhibition (50).

Class I PI3K phosphorylates a group of phospholipids known as phosphatidylinositol (PIs), so named for their inositol sugar head groups. PIs are a key component of all mammalian plasma membranes. Class I PI3Ks uniquely phosphorylate Phosphatidylinositol (4,5) bis-phosphonate (PIP$_2$), at the ‘3’ carbon position, thereby creating Phosphatidylinositol (3,4,5) tri-phosphonate (PIP$_3$) (51). PIP$_3$ is a key regulator of several downstream intracellular pathways, which it mediates via its binding sites for plextrin homology (PH) domains. PH domains are common conserved binding motifs found in many signaling proteins, which act to localize the molecules that contain them to specific cell compartments or membranes (51).

These motifs represent the molecular mechanism by which PI3K regulates multiple intracellular signaling pathways and circuits, including the mTOR pathway (50). Both PDK1 and Akt, contain PH domains (51). Once both molecules are brought into
close contact with each other via PIP₃ binding, PDK1 phosphorylates Akt at an activation site, Threonine-308 (52). This results in downstream activation of the mTOR pathway.

**Akt: Protein Kinase B**

Akt (Protein Kinase B; PKB) is a class of serine/threonine kinases that play central roles in the regulation of multiple intracellular signaling pathways, including the activation state of the mTOR pathway. Akt causes mTOR activation indirectly, through the phosphorylation and inactivation of two molecules that constitutively inhibit mTOR complex 1 (mTORC1) activity, PRAS40 (proline-rich Akt substrate of 40 kiloDaltons’) and the tuberous sclerosis complex 2 (TSC2) (see Figure 12; (53)). Dysregulation of Akt signaling or expression has been shown to promote metastasis and oncologic disease progression, either through attenuation of this mechanism, or any of the myriad other functionalities of Akt (54).

**mTORC1 and mTORC2: central mediators of mTOR pathway activity**

The mTOR pathway’s major downstream effectors are two similar serine/threonine kinases, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Both complexes contain mTOR itself (Figure 9). mTORC1 regulates protein synthesis globally, and couples cell size to cell cycle progression, while mTORC2 regulates cytoskeletal dynamics (47). Down-regulation of mTOR expression equally
affects the functioning of both of these complexes. Importantly, mTORC1 is susceptible to inhibition by Rapamycin, while mTORC2 is resistant to such inhibition. mTORC1 activation controls the downstream regulators of protein translation 4E-BP and P70S6K (47). mTORC2 is not involved in regulating these proteins; instead mTORC2’s major function is thought to be activation of Akt via phosphorylation at Serine-473 (52).

![Diagram of mTOR complexes 1 and 2](image)

**Figure 9. Schematic compositions of mTOR complexes 1 and 2. mTOR is a key component of both complexes.** mTORC1 uniquely contains Raptor and Pras40, and mTORC2, comprised of Rictor, Protor, and Sin1. Note that Rapamycin, while conjugated to FKRP-12, selectively inhibits the activity of mTORC1 and not mTORC2. Image taken from Bhaskar and Hay, 2007 (47).

**Dysregulation of the PI3K/Akt/mTOR Pathway**

Overexpression of the components of the mTOR pathway, particularly PI3K, have also been shown to be involved in the pathogenesis of multiple diseases. These include diabetes, cardiac hypertrophy, degenerative diseases, and cellular senescence (44, 45, 55).
PI3K is one of the most commonly mutated oncogenes in all of cancer biology, and its dysregulation has been implicated in the progression of a wide range of cancers, including breast, colon, endometrial, hepatocellular, and certain lymphatic and leukemic neoplasias (45, 53). The most direct causes of aberrant PI3K signaling are 1) loss of the tumor suppressor gene PTEN, which is a major inhibitor of PI3K activity, 2) activating mutations of either the α or β isoforms of the p110 catalytic subunit, or 3) activating mutations in any one of many growth factor receptors that regulate PI3K activity (50). Furthermore, many types of cancer are caused by the loss of tumor suppressor proteins, such as p53 or APC, which constitutively inhibit mTOR components, and thus indirectly cause an increase in their expression levels (55). Not surprisingly, the search for specific and potent inhibitors of members of the PI3K/Akt/mTOR pathway has been the focus of considerable interest by researchers in recent years (56).

mTOR Pathway Inhibition

Inhibition of the mTOR pathway at multiple levels has important consequences. One well-known problem in rational mTOR inhibitor design is that inhibition of mTORC1 alone causes a feedback activation of PI3K/Akt upstream signaling (57). The feedback activation of PI3K and Akt, resulting from selective mTORC1 inhibition, may counter the potential therapeutic benefits of such inhibition (57). This may explain the only modest effectiveness of rapamycin in treating human disease (sirolimus, and its derivative everolimus, for which the mTOR pathway was originally named), and explain its cytostatic rather than cytotoxic effects in some animal models (57, 58).
Similarly, feedback activation has been noted during the targeted inhibition of PI3K, using the PI3K-selective inhibitors wortmannin (irreversible) and LY294002 (reversible) (59). Such positive feedback activation may be the result of decreased expression of PS6K, which is engaged in constitutive negative feedback of PI3K. Perhaps as a result of this mechanism, both of these compounds have shown serious cytotoxicity in animal cellular models, and have consequently not been advanced to human clinical trials (59).

The extent of such feedback loop involvement may vary widely across the spectrum of cancers and virologic infections induced by PI3K dysregulation, and researchers must be careful about just how they go about inhibiting and manipulating this pathway. mTOR dysregulation (inhibition as well as stimulation) may up-regulate other potentially oncogenic cell signaling pathways, and thus affect levels of intracellular signaling molecules in unpredictable ways. For example, selective mTORC1 inhibition has been shown to activate the MAPK pathway, which has long been known to promote uncontrolled cell growth (60).

Further research into the biochemical and cell-signaling basis for how aberrant cell growth is induced, in an infectious or neoplastic setting, may help clinicians make point-of-care decisions about which combinations of PI3K/mTOR inhibitors (i.e. pan-PI3K, isoform-specific PI3K, dual mTOR/PI3K, or mTOR-specific) to give to patients (61). Ultimately, more well-informed treatment decisions will lead to better disease
management and control, and a better quality of life for patients who are afflicted by diseases or viruses involving mTOR pathway dysregulation.

**BK Viral Upregulation of the mTOR Pathway**

Recent studies have shown that during active infection, BKV upregulates the expression of multiple components of the PI3K/Akt/mTOR pathway (Figure 10; (22)):

![Figure 10. Sites of BKV activation and targeted pharmacological inhibition of the mTOR pathway.](image)

Green arrows indicate direct BKV upregulation: phosphoinositide-dependant protein kinase 1 (PDK1), Akt (PKB), mTOR, and the downstream mTOR effectors 4EBP-1 and PP2A.
p70S6K. Note that mTORC2 interactions are not shown. Sites of targeted pharmacological inhibition of the mTOR pathway are also shown; dactolisib (BEZ-235) is in purple, leflunomide and sirolimus are in red. The latter two drugs are commonly used immunosuppressive medications given to renal transplant recipients. Leflunomide is a targeted inhibitor of Akt and PDK-1, and possesses both antiviral and immunosuppressive activity (62). Sirolimus, the commercial name of rapamycin, for which the mTOR pathway is named, directly inhibits mTOR complex 1 (mTORC1). Ovals or perpendicular lines indicate inhibition, arrowheads indicate activation. Modified from Liacini, et al., 2010 (22).

BK-induced stimulation of the mTOR pathway causes, among many other effects, (1) increased phosphorylation (and activation) of the downstream mTOR effector p70S6 kinase (P70S6K; whose major role is as an upstream regulator of global levels of protein translation in the host cell), as well as (2) increased phosphorylation of the binding factor 4E-BP (22). Hypophosphorylated 4E-BP constitutively binds to and inhibits the protein eIF4E (elongation Initiation Factor 4E; Figure 11, (30)). mTOR activation induces FRAP-mediated phosphorylation of 4E-BP, promoting its dissociation from eIF4E, and binding of the latter to the 5’ cap of cytosolic mRNA (Figure 11). This eIF4E binding step must occur in order for the mRNA to be recognized by ribosomes and for translation to proceed (Figure 11; (63)).
Collectively, these two mechanisms increase the rate of translation of many kinds of proteins in the host cell, including ribosomes themselves (ribosomal biogenesis). Theoretically, any increase in the number of ribosomes by mTOR stimulation allows BKV to replicate its genome in an increasingly efficient manner (22). In sum, the targeted activation of the PI3K/Akt/mTOR pathway by BKV represents an opportunity for mTOR pathway inhibitors to specifically reduce the rate of host cell protein translation, and thereby reduce viral replicative efficiency.
Choice of Targeted mTOR Pathway Inhibitor

In the face of the limited clinical success of naturally occurring mTOR inhibitors such as rapamycin (which was originally isolated from *Streptomyces hygroscopicus* in 1975, from a bacterium native to Rapa Nui, a.k.a. Easter Island), researchers have looked to rationally synthesized compounds for more effective PI3K pathway inhibition (64). The results are promising. To date, over 25 mTOR-selective, PI3K-selective, or dual-inhibitor compounds have been identified and synthesized commercially by pharmaceutical companies such as Novartis, Pfizer, Astrazeneca, Merck, and others, and are in the process of intensive *in vitro* testing in several cellular models (45, 50).

One such second-generation mTOR inhibitor is Dactolisib, trade name NVP BEZ-235, which is a dual PI3K/mTOR inhibitor manufactured by Novartis Pharmaceuticals. BEZ-235 can be taken orally as a pill, once a day (65). This drug has been shown to strongly and specifically inhibit several components of the PI3K/Akt/mTOR pathway, including mTOR, PI3K, and the DNA-dependent Protein Kinases (DNA-PK) (Figure 12; (44, 61)).
Figure 12. Overview of signaling intermediates involved in the (A) mTOR pathway and (B) DNA damage response pathway, including sites of pharmacological inhibition. Akt upregulates mTORC1 expression by phosphorylating and down-regulating the effectors PRAS40 (proline-rich Akt substrate of 40kDa) and tuberous sclerosis complex 2, both of which are constitutive inhibitors of mTORC1 activity (59).

BEZ-235 (dactolisib; red) causes direct inhibition of Phosphotidyl-Inositol-3-Kinase (PI3K) as well as mTOR complexes 1 and 2, as well as certain kinases involved in the DNA damage response: ATM and DNA-dependent protein kinases (DNA-PKs). Activation of the latter two targets by double-stranded DNA breaks induces cell cycle arrest and the DNA damage response. BKM120 (dark blue) is an ATP-competitive inhibitor of PI3K; KU63794 (light blue) is an ATP-competitive inhibitor of mTOR itself, thus it effectively inhibits both mTORC1 and mTORC2, of which mTOR is a part. KU57788 (brown) and KU55933 (purple) selectively inhibit DNA-PK and ATM, respectively, via an ATP-competitive mechanism. Figure modified from Shortt, et al., 2013 (45).
NVP BEZ-235 was originally designed as a potential targeted treatment for certain cancers caused primarily by PI3K-activating mutations, and is currently in Phase II clinical trials for certain indicated cancers (44, 65). Structurally, BEZ-235 is an aromatic nitroheterocyclic organic compound, with several benzene rings. This drug also contains an imidazole ring (Figure 13; (66)). Other drugs in the imidazole class of medications are used as antibiotics, to treat a wide variety of infections. These include the common hospital acquired bacterium *Clostridium difficile* (Metronidazole), as well as certain fungal, protozoan and anaerobic bacterial infections (67).

![Figure 13. Structural formula of the imidazole Dactolisib, trade name NVP-BEZ235.](image)

BEZ-235 has a molecular formula of C$_{30}$H$_{25}$N$_{5}$O, molar mass of 469.5g/mol, and is a dual-PI3K/mTOR inhibitor currently being tested in clinical trials for some advanced-stage cancers. It is being tested as a potential antiviral agent in this thesis project. Imidazole ring is marked in red brackets. Figure modified from (66).
The decision to use NVP BEZ-235 (Dactolisib) over other targeted mTOR pathway inhibitors reflects its unique characteristics, as well as its relatively broader inhibition of multiple mTOR pathway targets. These traits set BEZ-235 apart from other mTOR inhibitors, particularly mTOR-selective inhibitors such as everolimus or sirolimus, and make it an ideal candidate molecule for novel testing as an antiviral agent.

**Current Experimental Approaches to Studying BKV**

There is no animal reservoir for BKV; the virus is capable of establishing latent infection in only certain human cell types (renal epithelial cells, among others). As such, there is no animal model currently available for BKV study. This fact severely limits the scope and nature of the experiments that BKV researchers can carry out. Without access to an animal model, it is difficult to characterize, manipulate, and study immune responses to BKV infection. The ability to infect rats or mice, for example, with BKV would allow researchers to fully explore the interplay and dynamics of different immune system cells as they respond to a BKV infection (i.e. antigen-presenting cells and BKV-specific CD8+ T-cells). Currently, many clinical studies are retrospective or observational in nature.

The ongoing research in the Tan laboratory involves analysis of kidney transplant patients’ cellular adaptive immune responses against BKV over time (temporal changes in the proportion of the CD8+ BKV-specific T cell population, both before and after the implantation of the transplanted organ). Patient blood and urine samples are received at
regular intervals, and PBMCs are isolated and challenged with BKV. Tetramers of MHC-I molecules that artificially express BK-VP1 peptide are incubated with patient PBMCs, and then either analyzed via flow cytometry or intracellular staining techniques. The results of these experiments are then correlated with clinical outcome (development of BK viruria, viremia, or BKVAN), in an attempt to understand more about interactions between BKV and the host immune system.

BKV research at Beth Israel Deaconness Medical Center centers around the use of cellular models of BKV infection, incorporating ‘immortalized’ renal proximal tubule epithelial (RPTE) cell lines, particularly CCD-1105. Other studies have used the fibroblast cell line CV-1 for BK in vitro studies (23). Cell lines are said to be ‘immortalized’ if they are theoretically able to replicate indefinitely, when given proper growth conditions. The immortality of cellular cultures is often accomplished via genomic insertion of the SV40 Large T antigen or other oncogenic viral proteins (68).

This artificial genetic manipulation is relevant when staining for BK Large T antigen produced in virally infected host cells – particularly those cell lines immortalized with SV40 Large T antigen. Because no commercially-available monoclonal antibody for BK Large T exists, researchers instead use the cross-reactive polyclonal SV40 Large T antigen to stain for this BK protein, from Santa Cruz Biotechnology, CA. As might be expected, this polyclonal antibody may bind to SV40 Large T antigen produced at low levels endogenously in these cell lines. Results of assays involving BK virally infected cell lines that have been immortalized with SV40 Large T antigen must be interpreted carefully in order to minimize over-estimation of Large T expression. In order to prevent
this very problem, the cell line used in this project, CCD-1105 renal epithelial cells, were immortalized using oncogenic human papillomavirus 16 proteins [CCD 1105 KIDTr (ATCC CRL-2305™), ATCC, Mannassas, VA]. No cross-reactivity with SV40 Large T antigen antibodies occurs in this cell type.

Some studies suggest that renal tubule cell populations taken directly from animal or human tissues (‘primary cell lines’), rather those that have been artificially immortalized, provide more realistic intracellular environments for the study of BKV infection and replication (69). However, such cultures are more time-consuming to maintain, and ultimately do not provide the standardization, consistency, and reproducibility of immortalized cell cultures.

Aims of the Proposed Study

This thesis project is an attempt to evaluate the efficacy of the dual-PI3K/mTOR inhibitor NVP BEZ-235 (Dactolisib) as an antiviral agent, in a cellular model of BKV renal infection. It will attempt to show conclusively that BEZ-235 indirectly reduces the replicative efficiency of BK virus, via down-regulation of components of the mTOR pathway.

A drug toxicity screen was carried across a range of experimental BEZ-235 concentrations (0nM – 1000nM), using the MTT assay. Culture and preparation of cells for this assay followed an identical time-course and procedure to that of the subsequent experimental trials, to determine if the drug induced any significant degree of
cytotoxicity. Following positive findings from this screening (a lack of significant observed cytotoxicity), further experimentation was carried out.

Cells were grown according to established and standardized protocols, under sterile conditions. After plating, each well was ‘pre-incubated’ for 48 hours with appropriate concentrations of BEZ-235, and then infected with BK virus (or independently with SV40 virus, if indicated). Virus was then removed from the supernatant, and cells were allowed to grow in fresh media for a further 48 hours. One of three types of assays were then carried out to investigate the extent of viral replication: 1) quantitative PCR measuring number of viral genomes found intracellularly in each sample, 2) Western blot analysis for levels of proteins in the mTOR pathway or viral proteins, or 3) immunohistochemical staining for the number of Large-T-antigen expressing cells.
METHODS

Drug Toxicity Screening – the MTT Assay

As Paracelsus famously said in the early 1500s, ‘the dose makes the poison;’ this toxicological concept still rings true today (70). Any compound, even water, which most see as essential for life, can be toxic to the human body, given a dose of sufficient strength.

In order to begin testing the research question, it was first necessary to confirm that BEZ-235 was, in fact, not toxic to the cells themselves at the concentrations used. Drug-induced cell toxicity of any type represents a confounding factor that must be screened for prior to the start of any viral infection studies. This is especially true because the degree of BEZ-235 cytotoxicity is concentration-dependent, and all assays will be carried out on a wide range of drug concentrations.

Poisoned cell populations are likely to have overall dysregulation of metabolic activity, higher levels of stress-induced cytokines, and accumulation of lysed cellular debris in the extracellular space. Importantly, many of the major intracellular pathways will likely also be abnormally expressed, including the mTOR pathway, which is the major focus of this project. For all these reasons, drug-induced cellular toxicity may adversely affect cellular responses to BK infection, and potentially invalidate any and all results we might obtain from experimental assays.
There are several well-validated cell cytotoxicity assays that are commercially available. Most of these assays allow for quantitative determination of the ratio of live to dead cells in any given cell population. The Vybrant MTT Cell Proliferation assay (Molecular Probes - Life Technologies, Grand Island, NY), which is a rapid, easily performed colorimetric assay that measures metabolism of the salt dimethyl thiazolyl diphenyl tetrazolium (MTT) was used in this study (71).

Non-reduced MTT is a yellow compound, whose relative abundance in a cell can be approximated by spectrophotometer readings at 630nm. This compound is reduced enzymatically by mitochondrial succinate dehydrogenase (familiar to biologists as Complex II of the electron transport chain), into an insoluble purple product. The rate and overall relative abundance of the reduced and oxidized forms of MTT is dependent on the overall metabolic status of the cell (72). Dead cells will contain only non-reduced (yellow) MTT, and will thus not contribute to the intensity of purple color produced in a given well. Colorimetric purple intensity can be determined using a spectrophotometer measuring light produced at 570nm, and subtracting from that value any background signal, measured at 630 nm. Comparisons can then be made with this quantitative information, across conditions.

**Time-course of Culture of BEZ-235 Incubation and Viral Infection Protocol**

Following positive preliminary results from the MTT assay using appropriate concentrations of BEZ-235, it was next necessary to determine an appropriate time-
course of events during drug incubation and BK viral infection. This time-course was optimized and standardized at the beginning of the experimental period, and used as consistently as possible for all assays (Figure 14).

![Figure 14. Schematic of time-course of CCD-1105 drug pre-treatment and viral infection protocol for all experimental assays in this project. Note that glass chamber slides, rather than 6 well plates, were used for immunohistochemistry experiments. All other conditions were held constant.

1mL aliquots of CCD-1105 cells (containing 1*10^6 cells) were individually thawed in a water bath and plated with ~14 mL of K-10 growth media in a 175cm² flask. After 48 hours of growth, cells were... ]
plated in 6 well plates at 50K cells/well (exact cell counts were determined using a haemocytometer), and allowed to grow for a further 48 hours, with incubation of 3 mL of K-10 media containing appropriate BEZ-235 dilutions. Cells were then washed with PBS and infected with BKV (stored at -80 °C) at MOI of 1.0 for 3 hours. Wells were then washed with PBS and replaced with 3 mL of K-10 media. Cell growth was observed via light microscopy at all stages of the time-course. 175 cm² flask image taken from: http://www.sigmaaldrich.com/content/dam/sigma-aldrich/product5/020/cls3815.tif/_jcr_content/renditions/large.jpg

Standardization of CCD-1105 Population Life Cycles

Immortalized renal epithelial cells (CCD-1105 KIDTr, American Type Culture Collection (ATCC, CRL2305), Manassas, VA) were purchased, and arrived in the laboratory at 133 days of growth. They were then grown in sterile tissue culture conditions in 175 cm² flasks, using K-12 media (comprised of D-MEM R-10 base media (Gibco/Life Technologies, Grand Island, NY), 10% fetal bovine serum (Hyclone/Thermo Scientific, Waltham, MA), 1% gentamycin (Gibco/Life Technologies, Grand Island, NY), and 5% penicillin/streptomycin. ~80% plate coverage (confluency) was achieved after ~48 hours. At that time, cells were then ‘passaged’ into a new sterile flask at a ratio of 1:3.

Prior to transfer, cells were washed once with Dulbecco’s Phosphate Buffered Saline (dPBS; Life Technologies, Grand Island, NY), rendered non-adherent with 2.5 mL of trypsin (TryoLE Select, Gibco/Life Technologies, Grand Island, NY) and homogenized in ~12 mL of media. 4 mL (representing a one third of the total cell population) of this suspension was then transferred to a new sterile 175 cm² flask, and the cell population was allowed to settle and adhere to the flask surface. Thus, only one third
of the cell population was transferred to the new flask, so optimal cell growth could continue. The process was repeated until passage 8, when cells were frozen in 1mL aliquots of 1*10^6 cells at -130°C, for use in all subsequent BKV/BEZ-235 assays.

*Cell Plating, Growth, and BEZ-235 Incubation*

1mL aliquots of CCD-1105 cells were thawed as needed, and grown in 175 cm² flasks for ~48 hours. They were then plated in 6-well plates at 50,000 cells per well (see Figure 14). BEZ-235 drug treatments included 1000nM, 100nM, 10nM, and 0nM, as well as wells for MOI (‘Multiplicity of Infection’) determination and an SV40 positive control when appropriate (IHC assays only; explained below).

After 48 hours of growth, media was aspirated (via vacuum aspiration), cells were washed once with PBS, and BEZ-235 was added at appropriate concentrations in 4 mL of media in 6-well plates (Figure 15).
Figure 15. Diagram showing the drug incubation conditions for cell populations contained in a six-well plate. The well for MOI determination was located in a separate plate, to minimize unnecessary handling and prevent contamination of the remaining wells.

**Multiplicity of Infection (MOI) Determination**

Following 48 hours of BEZ-235 incubation, a cell count used to determine MOI was performed, and BK viral infection was carried out at an MOI of 1.0 for three hours. MOI is a quantitative ratio set by the researcher (usually 1.0, .1, .01, etc) used to standardize the number of infectious agents (bacterial, viral, or otherwise) added to a population of host cells, across experiments or research groups. This is important because cell populations grow at slightly different rates, even when all other conditions are held constant (number of cells plated, incubator humidity and CO₂%, volume of cell culture media, etc). As a result, adding a constant number of virions to every experimental trial may not produce the same number of infectious events. On a cellular level, some CCD-
1105 cells may be challenged by three or more BK virions, some by none at all. By
determining the approximate number of host cells present in the well immediately prior
to viral infection, and adding an identical number of virions (for MOI of 1.0), researchers
can most accurately standardize the viral infection process, and thus reduce error.

Cell counting was performed by trypsinizing and subsequently resuspending
cells from a single well, in 1mL of media. 10 uL of cellular suspension was mixed via
pipette with 90 uL of Trypan Blue, a cellular visualization agent (Gibco/Life
Technologies, Grand Island, NY), creating a 1:10 dilution. 10uL of this dilution was then
pipetted into plastic hemocytometers (Hycor Biomedical, Indianapolis, IN), which
contain small grids comprised of 1mm by 1mm squares into which cellular suspensions
can be injected. Live cells were systematically counted at 100x magnification, and
independent counts in four of these squares were averaged and used to estimate the total
number of cells contained within a 1 mL suspension. Specifically, this estimation was
calculated using the following equation:

\[
\frac{XmL[AveragedHemocytometerGridCellCount + 10 \times 10,000]}{XmL}
\]

Here, ‘X’ is the total volume of cellular resuspension, generally 1 or 2 mL. ‘10’
represents the 1:10 Trypan Blue dilution, and ‘10,000’ accounts for conversion factors
used in representing the volume of 1:10 dilution contained within a single ‘square’ of the
hemocytometer.
Endpoint Assays for Measuring Efficiency of Viral Replication

Upon the completion of 48 hours of further growth post-viral infection, all samples were centrifuged in 1.7mL microcentrifuge tubes, pelleted (via aspiration of the supernatant), and frozen at -20º C. Samples were then thawed at room temperature immediately prior to analysis by one of three approaches: extraction of DNA followed by quantitative (real-time) Polymerase Chain Reaction (qPCR), analysis of protein content by Western blot, or Fluorescent protein staining with immunohistochemical techniques.

Fluorescent Real-Time Polymerase Chain Reaction

PCR is an attempt to quantify the exact amounts genetic material (from virus genomes as well as host cell genomes) present in each sample. Because genetic material is potentially present ubiquitously on all surfaces in the lab environment, it is imperative that strict cleaning protocols are followed. Lab coats, lab benches, reagents and pipette tips were stored separately and specified as ‘DNA extraction only’ or ‘PCR only.’ These materials were not used for other assays or by other laboratory workers. Prior to extracting DNA from frozen whole cell samples, all pipettes, tube racks, and any other supplies used in the biosafety cabinet were sprayed (with subsequent drying after each step) with 70% ethanol, then DNAZap (Ambion/Life Technologies, Grand Island, NY), then twice more with 70% ethanol.
DNA extraction from experimental samples was carried out as per instructions contained within the Qiagen DNA Blood Mini Kit (Qiagen, Valencia, CA). Briefly, samples were lysed and run through a series of centrifugations with different lysis and wash buffers, as well as 95% ethanol, which efficiently precipitates the DNA. After the precipitated DNA had been washed sufficiently, 200 uL of a final buffer (Buffer AE) was added to dissolve the DNA and allow it to be collected in a microcentrifuge tube. The tubes were then frozen at -20 °C while PCR reagents were being prepared.

All PCR reactions were carried out in 96-well plates optimized for use in a PCR machine (MicroAmp, Applied Biosystems, Bedford, MA), with each sample and standard curve well (discussed below) being run in triplicate. 20 uL of each sample were added to 30 uL of ‘Master Mix,’ (TaqMan Universal Master Mix, Applied Biosystems/Life Technologies, Grand Island, NY), for a total reaction volume of 50 uL.

The Master Mix contained everything needed to carry out 40 consecutive cycles of Fluorescently quantifiable DNA amplification: 80 uL each of (1:20) BK VP1-specific forward (5'-AGTGGATGGGCCAGCCTATGTA-3') and reverse (5'-TCATATCTGGGTCCCCTGGA-3') primers (Invitrogen). Five uL of Fluorescent probe (6FAM-AGGTAGAAGGTTAGGTGGTTTGATGGAACA), also specific for the same VP1 sequence, containing both ‘reporter’ and ‘quencher’ dyes, Taq Polymerase (whose exact volume depended on the number of sample wells being run), an excess of nucleosides with which to create the newly synthesized DNA, and 335 uL of double distilled water. This mixture was pulse-vortexed and pipetted into the 96-well plate, and
then covered with a clear plastic sheet to prevent contamination. The plate was then refrigerated at 4 °C while standard curve dilutions were prepared.

The ‘standard curve’ is a line-of-best fit that must be calculated anew with each and every PCR amplification of an experimental sample. This curve is generated using a serial dilution of a known number of genetic components: $10^6$, $10^5$, $10^4$, $10^3$, $10^2$, $10^1$, 5, and 0 copies. Care was taken to reduce any potential cross-contamination of genetic material between these dilutions as they were being made. The appropriate volumes of ddH$_2$O were prepared prior to any plasmid being added to the dilutions. Microcentrifuge tubes containing any concentration of plasmid were opened with one arm underneath the lab bench, and closed in a similar position, to reduce aerosol contamination. The genetic material used to generate the standard curve in these PCR assays was a plasmid, pBR322, containing a full-length insertion of BKV (provided by the Koralnik Lab). The standard curve is needed for the PCR program (7300 System Sequence Detection Software [SDS], v. 1.2.3, Applied Biosystems) to accurately ‘back-calculate’ and interpret florescence data measured sample amplification in the thermocycler.

Once prepared, serial pBR322 plasmid dilutions were briefly vortexed to homogenize the solution, and 20 $\mu$L was loaded onto the 96-well plate in triplicate. Experimental wells remained covered during the dilution plating process. A new plastic cover sheet was added and the entire plate was centrifuged at 2000 rpm for 2 minutes, to remove any droplets on the sides of wells. Samples were then placed in the 7300 Real-Time PCR (Applied Biosystems), and run through 40 amplification cycles (described in the text of Figure 5).
Western Blot Protein Assays

Samples were thawed to room temperature and lysed in 200μL of lysis buffer (Cell Signaling, Danvers, MA), containing a 1:100 dilution of protease/phosphatase inhibitor cocktail (Cell Signaling, Danvers, MA) on ice for 15 minutes. Protease/phosphatase inhibition was needed because activated mTOR pathway proteins are phosphorylated at specific residues, and the state-dependent nature of this pathway is likely intensely dysregulated during the cells’ stressful period of cell lysis. Specifically, lysis of all host cell membranes will cause all cellular compartments (lysosomal, nuclear, endosomal, Golgi Apparatus, etc) and their contents to merge, including any proteases or phosphatases contained within each compartment.

After incubation on ice, samples were then centrifuged at 8,500 rpm for 10 minutes in the cold room. 12μL of sample was then combined with 12μL of loading buffer. Loading buffer was prepared by mixing Laemmli buffer (BioRad Laboratories) with 5% β-mercaptoethanol (BME; Sigma-Aldrich, St Louis, MO). BME is used in Western blotting applications to denature protein quaternary structure and prevent polymerization, which may affect gel migration rate and subsequent band formation. This mixture was then denatured in a heated water bath for 10 minutes. Samples were then allowed to cool to room temperature, and centrifuged briefly to remove droplets from lid.

10% polyacrylamide pre-cast gels (Mini-PROTEAN TGX, BioRad, Hercules, CA) were immersed in running buffer (1:10 dilution of 10x Tris-Glycine SDS buffer
(BioRad) and placed in the gel apparatus (BioRad) prior to sample loading. A protein standard was also included in all gel runs (Precision Plus Protein Kaleidoscope Standard, BioRad) to allow for band size identification.

Electric current was run across samples at constant voltage (100V; ~40mA) using a power source, for approximately 60-75 minutes, or until samples had run the length of the gel. Protein content was transferred from gel to membrane using an automated system (iBlot Transfer System, Invitrogen, Carlsbad, CA).

The membrane was cut to size, washed for ten minutes twice in 0.05% Tween Tris-Buffered Saline (TBS-T) solution (BioRad), and blocked in a 5% bovine serum albumin-TBS solution for one hour at room temperature, on a rocker. Both Tween and bovine serum albumin (BSA) are included in these washes as blocking agents; to reduce the non-specific binding of antibodies to proteins other than the target sequence. The membranes were then washed three more times (ten minutes each) with TBS-T, and incubated with rocking overnight in a 1:1000 dilution of ‘primary’ antibody in 3% BSA-TBS solution at 4° C. The ‘primary’ antibody, is by convention specific for the target of interest, but is otherwise unable to be visualized. The addition of a ‘secondary’ antibody is required to detect the protein of interest.

Primary antibody was then poured off, and the membranes were washed twice with TBS-T, and once with TBS, on a rocker at room temperature. A solution of 3% BSA in TBS-T, with ‘secondary’ antibody (at 1:1000 concentration), was then prepared and incubated with the appropriate membranes for one hour at room temperature, on a rocker.
The ‘secondary’ antibodies used in this study were conjugated with a specific enzyme, horseradish peroxidase, which cleaves its specific substrate to produce detectable levels of a luminescent signal. By convention, secondary antibodies are produced by the immune system of an animal or host distinct from that which produced the primary antibody that was used in the experiment. All mTOR pathway–specific antibodies used in this project were rabbit immunoglobulins, meaning they were produced and harvested from rabbits (Cell Signaling Technologies, Danvers, MA). Thus, for these proteins specifically, we used HRP-conjugated mouse anti-rabbit monoclonal antibodies as ‘secondary’ antibodies. Doing so allows us to most effectively visualize any primary antibody used to target proteins, and produce the most accurate signal pattern during development.

After secondary antibody incubation, membranes were washed three times with TBS-T, and twice with TBS, for ten minutes each on a rocker. Immediately following this final wash, they were incubated for 5 minutes in ECL developer solution (Pierce Products, Thermo Fischer Scientific), and film negatives were produced in a dark room. Multiple exposures were produced to obtain the optimal image quality.

Immunochemistry (IHC) Staining

Because imaging of fluorescently stained adherent cells is accomplished directly in the container in which the cells are grown, glass chamber slides (Nunc™ Lab-Tek™ II Chamber Slide™ System, Thermo Scientific), rather than 6 well plates, were used in the
IHC protocol. The smaller volume in these chambers also necessitated other small changes from the ‘standard’ experimental protocol: 500 μL of media were used during viral infections, and 1 mL of media was used per well, for all growth incubations.

Upon completion of the 48-hour post viral infection growth period, glass chamber slides were washed twice with 1 mL of PBS. Cells were then fixed via incubation with a 4% formaldehyde solution (Polysciences, Incorporated, Warrington, PA), for 20 minutes at 4°C. The samples were brought from the tissue culture room to the wet lab, where chamber ‘walls’ were removed (leaving only glass microscopy slides). Slides were permeabilized with .2% Triton solution (Sigma Aldrich, St Louis, MO), for 10 minutes at room temperature, in a humidified chamber. Cells were washed twice in PBS in glass histological wash containers, each for 5 minutes.

Slides were blocked with a 5% normal goat serum (NGS) solution of PBS, (Vector Laboratories, Burlingame, California), for 15 minutes. Goat serum, like serum from all species, contains all the protein components of blood – including albumin, which reduces nonspecific antibody binding by ‘collecting’ the majority of proteinaceous material in the sample.

Slides were then incubated for two hours in a humidified chamber with primary antibody against SV40 Large T antigen (SV40 T Ag v-300, Santa Cruz Biotechnologies, Inc, Santa Cruz, CA), at a concentration of 1:200 in 5% NGS, at room temperature. Primary antibody was then tapped off onto a paper towel, and slides were washed in wash buffer and PBS, for 5 minutes each, to remove background staining. Slides were rinsed a final time in ddH₂O, mounted using Vectashield with DAPI (DAPI is commonly used as a
nuclear stain; Vector Laboratories, Burlingame, CA), and then imaged using a Fluorescent microscope. Positive DAPI and Large T antigen counts per field were determined using the computer program ImageJ. This program detected point maxima in DAPI- and Large T-stained cells (‘Process’ → ‘Find Maxima..’ → ‘Tolerance = 28’). Each point is assumed to represent a unique positive staining event.

*Spectrophotometer Absorbency Reading*

Absorbency readings at various wavelengths can provide specific information about the concentration of certain compounds in a solution. Because double-stranded DNA absorbs UV light at 260 nm, and protein contamination can be approximated via absorbance at 280 nm, we calculated the ratio of these two absorbencies using a spectrophotometer (Nanodrop ND-1000 Spectrophotometer, Thermo Fisher Scientific, Waltham, MA; (73)).

Extracted DNA samples were thawed to room temperature and 10 µL was aliquoted into separate labelled microcentrifuge tubes, along with a 10 µL aliquot of the elution buffer used in the extraction itself, AE buffer (Qiagen). These aliquots were then brought to the spectrophotometer for analysis; the remaining volume of extracted DNA was returned to the -20°C freezer for storage.

The device was first ‘cleared’ by loading and analyzing a 2 µL sample of ultrapure deionized water (Invitrogen, Life Technologies, Bedford, MA). Next, background signal was minimized by ‘blanking’ the machine with 2 µL of AE buffer, the
solvent into which samples were extracted. Between each sample reading, all surfaces that had liquid were blotted dry with a KimWipe (Kimberley-Clark Professional, Irving, TX), wiped with deionized water on a second KimWipe, and vigorously rubbed dry. Measurements across all BEZ-235 concentrations were repeated in triplicate.
RESULTS

Observational Data

One important observation made while analyzing cell growth during BEZ-235 incubation, was that in many of the 1000nM wells, and to a much lesser degree in 100nM and 10nM wells, small crystalloid fragments were observable via light microscopy. These fragments were not visible in 0nM wells, and were non-adherent and present in multiple focal planes, making it likely that BEZ-235 had precipitated out of solution. Further experimentation using 1000nM solutions revealed that they were present in higher numbers in certain liquids than others (double distilled H2O > K-10 media > PBS; data not shown). Collectively, these data suggest that 1000nM is not an appropriate physiological dose, and that results from this experimental condition should be interpreted with caution.

MTT Assay

The results of the MTT cell viability assay were as follows:
Figure 16. MTT CCD-1105 cytotoxicity assay performed in a 96-well plate following 48 hours of BEZ-235 pretreatment and ‘mock’ viral infection protocol conditions (100µL of media alone was used), as outlined in Figure 14. Cells were initially plated at 4,900 cells/well. Values shown are the averaged difference in absorbance readings (570nm–630 nm) of wells plated in triplicate, across all treatment conditions, with standard error bars shown. A set of ‘blank’ wells, containing MTT alone and no cellular component, were included. Three wells were also pre-treated with 3% formaldehyde, 20 minutes prior to MTT addition, to induce cell death as a positive control condition. All treatment conditions are not significantly different (one-way ANOVA, p > .05; analysis not shown).

Efforts to replicate identical conditions in the MTT assay protocol to those of the experimental time-course were stymied by technical limitations. The spectrophotometer (SpectraMax 384Plus, Molecular Devices) was only able to read 96- or 384-well plates, not the 6 well plates that were used for all experimental assays. This represents a small but necessary deviation from an exact replication of the protocols of the ‘master’ experimental time-course. The number of cells plated was scaled down according to the
ratio of each plate’s surface area, to preempt cellular overcrowding: [9.4 cm²]:[.32 cm²] gives a numerical ratio of ~30x. Thus, the original cell population of 50,000, plated in 6-well plates, was reduced to (50,000/30) = ~1,700 cells per well for plating into 96-well plates.

**Quantitative PCR**

qPCR analysis revealed variable and somewhat inconclusive results, when probing for a relationship between BK viral copy number and BEZ-235 drug treatment concentration (Table 2; Figure 17). qPCR analysis was conducted as described above, and BKV copies/PCR reaction were calculated (‘#’ in Table 2). This information was then correlated with the mass of total DNA loaded per reaction via spectrophotometer analysis at 230nm, in order to calculate copies BKV/ug DNA (see Table 2). Results are shown graphically across BEZ treatment conditions in Figure 17.

**Table 2. Tabulated values for quantitative PCR and spectrophotometer assays, showing calculation of copies BKV/µg DNA.** ‘#’ represents ‘raw’ numerical data produced after PCR amplification of VP-1 specific sequences of BEZ-235 treated cell populations. Because 20 µL of sample were added to each PCR reaction well, BKV copies/µL column was calculated by dividing ‘#’ by 20. ‘*’ represents raw spectrophotometer readings, reflecting the measured concentration of all DNA (host cell and BK viral), in ng/µL. Dividing this column’s values by BKV copies/µL results in the cancellation of µL, and gives copies BKV/ ng DNA. The final column (far right) shows copies BKV/µg DNA; these values are shown graphically in Figure 17.
<table>
<thead>
<tr>
<th>BEZ Concentrations</th>
<th>viral copies/PCR reaction #</th>
<th>viral copies/µL</th>
<th>Ng DNA/µL *</th>
<th>Copies BK/ng DNA</th>
<th>Copies BK/µg DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 nM</td>
<td>28100000</td>
<td>1405000</td>
<td>55.9</td>
<td>25138</td>
<td>25</td>
</tr>
<tr>
<td>10 nM</td>
<td>4720000</td>
<td>236000</td>
<td>7.4</td>
<td>31827</td>
<td>32</td>
</tr>
<tr>
<td>100 nM</td>
<td>11366667</td>
<td>568332</td>
<td>44.4</td>
<td>12809</td>
<td>13</td>
</tr>
<tr>
<td>1000 nM</td>
<td>17066667</td>
<td>853332</td>
<td>6.9</td>
<td>123092</td>
<td>123</td>
</tr>
</tbody>
</table>

**Figure 17.** Copies of BK virus per µg of total CCD-1105 DNA, across treatment with variable BEZ-235 concentrations. Ratios were calculated using primers specific for BKV VP1, in a quantitative (‘real-time’) Fluorescent PCR assay. These concentrations spanned a relatively wide range: 0, 10, 100 and 1000 nM.
Western Blot Assays

In Western blotting, it is standard to include a ‘loading control’ assay for each experimentally assayed protein. Usually, loading control proteins are present ubiquitously in the intracellular environment of most cell types, such as the cytoskeletal proteins actin or tubulin. A similar loading control signal across wells ensures that one well was not loaded with more sample than the others.

When possible, we sought to measure the phosphorylation status of mTOR pathway proteins as a function of their total concentrations in the cell. Thus, rather than use a ‘standard’ loading control protein such as tubulin or actin, for the Akt-Ser473 assay, a pan-Akt antibody was used (Figure 18).

Antibodies used in mTOR pathway Western blotting were chosen for the functional significance of the residues that they were specific for. Full Akt kinase activity occurs when both serine 473 and threonine 308 are phosphorylated, although the relative importance of each site is a subject of debate (74). A key phosphorylation site on mTOR is at residue serine 2448, which is the phosphorylation site that activated Akt indirectly upregulates (via decreased PRAS40/TSC2 phosphatase activity) (75). Phosphorylation of all of these residues represents a dynamic equilibrium between phosphatases tending to reduce phosphorylation, and kinases tending to promote phosphorylation. We examined the status of these mTOR pathway markers via Western blotting, in both the presence and absence of BK infection. In BKV-infected cells (MOI=1.0), we saw a dose-dependent decrease in Akt Ser-473 phosphorylation, indicating decreased Akt activity (Figure 18).
Furthermore, a strong trend of decreased BK Large-T antigen expression was seen with increasing concentrations of BEZ-235 pretreatment (Figure 19). A weaker trend of decreased mTOR phosphorylation at serine residue 2448 was observed in non-virally infected cells (Figure 20). The implications of these results will be discussed below.

![Akt-Serine 473 diagram](image1)

**Figure 18.** Treatment of BK-infected CCD-1105 cells with the dual PI3K/mTOR inhibitor BEZ-235 reduces activation of the mTOR intermediate Akt, as measured by phosphorylation at the Serine-473 phosphorylation (Akt MW is 60kD). SV-40 (strain 776; provided by X. Dang) was used as a positive control. The ladder did not visualize upon film development, but was visible on the undeveloped membrane itself. The two images were superimposed and the ladder was drawn on the film in sharpie, to assist in sample size identification.
Figure 19. Treatment of BK-infected CCD-1105 cells with the dual PI3K/mTOR inhibitor BEZ-235 reduces efficiency of BKV viral replication, as measured by Large T antigen expression. BK Large T antigen expression (MW 90kD, cross-reactive with the primary antibody used in this assay, SV40 Large T antigen) was measured against basal levels of Akt (pan-Akt; MW 60kD). SV-40-infected cells (strain 776; provided by X. Dang) were used as a positive control.
Figure 20. Treatment of non-infected CCD-1105 cells with increasing concentrations of the dual PI3K/mTOR inhibitor BEZ-235 modestly reduces the phosphorylation of mTOR at residue serine 2448 (MW is 249kD). α-tubulin (40kD) was used as a loading control.
Fluorescent Immunohistochemical Analyses

Fluorescent immunohistochemical staining analyses of BK Large T antigen and DAPI expression in BKV infected cells showed promising results. A preliminary visual inspection of randomized fields of view in each of the treatment conditions appeared to show a net decrease in the number of infected cells, when compared to the total number of cells per image (i.e. both productively and nonproductively infected; Figure 21). These visual observations were confirmed by a quantitative analysis (Figure 22). The ‘raw’ positive counts across conditions, used to generate Figure 22, are shown in Table 3.

Figure 21. Representative immunohistochemical staining images across five BEZ-235 concentrations: 0 nM, 10 nM, 100 nM and 1000 nM. Also included was an identical well infected with SV40 as a positive control to account for use of SV40 Large T polyclonal antibody (Santa Cruz Biotechnologies, Dallas, TX). DAPI/Large T images taken from an identical field of view via Fluorescent microscopy at 200x magnification. Scale bars represent 50 µm.
Table 3. Positive counts of A) DAPI- and B) Large T-antigen stained, BKV-infected, CCD-1105 cells. Counts were tallied per field of view of (200x magnification), and averaged over five randomized images per treatment condition. Images were analyzed visually for Large T antigen stained images, and using ImageJ (NIH) for DAPI-stained images. Green boxes contain averaged values; ‘SV40’ column contains cells infected with SV40 rather than BKV. Calculations for standard deviation and standard error are also shown.

<table>
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<th>A) DAPI Positive Counts</th>
<th>Condition</th>
<th>BEZ235</th>
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<table>
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</table>
Figure 22. Percentage of BK Large T-Positive nuclei to DAPI-positive nuclei in immunohistochemical staining assay of BKV-infected CCD-1105 cells (values taken from green-colored cells in Tables 3A and B), with 48-hour BEZ-235 treatment at the indicated concentrations. Ratios reflect averaged values of sets of five randomized slide images (shown in Table 3 above), at 200x magnification. Two identical trials of the IHC were performed, with similar quantitative results (data not shown). Bars with ovals indicate statistically significant differences (one-way ANOVA; ‘o’ indicates p<.05).
DISCUSSION

The goal of this project was to quantify the effects of the mTOR pathway inhibitor BEZ-235 on BK viral replication, using three separate biochemical approaches. By having each assay substantiate and confirm the results of the others, rather relying only on the strengths (and weaknesses) of a single assay, the hope was that we would be more certain of the effect of this compound on virally infected CCD-1105 cells.

The combined results of these three sets of assays, particularly the Western blot examining Akt Serine-473 phosphorylation status, suggest that BEZ-325 effectively reduces mTOR pathway activity in a reproducible manner. This is consistent with what past studies have demonstrated using this drug in similar cell lines, as well as being its pharmacological raison d'être.

The reality, however, is that regardless of how efficiently BEZ-235 down-regulates the mTOR pathway in uninfected host cells, the result of its effects on mTOR pathway activity is much more nuanced in BKV-infected cells. The ‘net outcome’ in such experimental conditions is likely dependent on the summation of BEZ-235-induced downregulation and BKV-induced upregulation of this signaling pathway. Other potentially relevant factors that may regulate the observed activity of the mTOR pathway include the number of virions added per well, the host cell density (see qPCR discussion for further details), BEZ-235 metabolism and pharmacokinetics, and the duration of BEZ-235 treatment. Regarding the effect of the duration of drug treatment, it was unclear whether 48 hours was sufficient to promote adequate mTOR down-regulation. Should
BEZ-235 have been added after viral infection for a further 48 hours? Further work will be required to address this question.

Given the observed BEZ-235 precipitation in 1000nM wells, it was likely that the range of BEZ-235 concentrations tested was broader than necessary. Given that BEZ-235’s IC$_{50}$ (concentration at which 50% of the indicated enzyme activity is abolished) is ~4 nM for PI3Kα, and 20.7 nM for mTOR itself, further studies should perhaps use BEZ-235 concentrations closer to those levels (76). Nonetheless, our use of higher concentrations of BEZ-235 on intact cells was a reflection of the fact that the EC$_{50}$ of this drug for inhibition of mTOR signaling is clearly higher than the IC$_{50}$ observed when examining effects on purified enzyme.

Of note is that mTOR inhibition, of any potency, does not interfere with BK viral entry into the host cell. BEZ-235 does not block BKV binding to ganglioside host cell receptors, nor does it affect any step in BKV vesicular trafficking to the host cell nucleus, for that matter. As such, the rate of viral entry across all BEZ-235 assays can be presumed to be equal. The results of MTT assays indicate that there is likely little toxicity within the range of BEZ-235 concentrations tested. However, given the differential DAPI-positive cell counts during the IHC assay (see Table 3A), it is likely that BEZ-235 may produce a cytostatic effect by reducing the rate at which cells progress through the cell cycle.
MTT Assay

The conclusions that can be drawn from this cytotoxicity assay, shown in Figure 16, are limited. It is clear, however, that because there are no significant differences across treatment conditions, the range of absorbance readings across all treatment conditions is similar, and BEZ-235 does not, at least in this assay, cause an observable cytotoxic effect at the concentrations and tested.

Interestingly, the highest absorbance readings, which supposedly correspond to a larger quantity of ‘yellow’ MTT being reduced (metabolized) into ‘purple’ product by live host cells, were found in wells incubated with 10 nM BEZ-235 and 3% formaldehyde. This finding is the opposite of what might be expected, and contradicts the supposed cytotoxic nature of formaldehyde, which is commonly used as a cellular fixative.

There are likely several confounding factors that influence the absorbency readings of the MTT assay. It is possible that, due to the small number of cells/well (10 – 20,000 at the time of assay), relatively small quantities of MTT were metabolized and that the spectrophotometer lacks the sensitivity/resolution necessary to detect such small differences. Homogenization and resuspension of the solution after MTT metabolism produced air bubbles in virtually all wells. The presence of these air bubbles likely affected the accuracy of measurements across all drug concentrations, and may have produced the trends seen in Figure 16. Other potential factors affecting the results of this assay include the use of multiple aspiration steps (while BEZ-235 was added and removed, wash steps, etc) and the small number of cells (5,000) plated per treatment
condition. Given the sensitivity of this assay, loss of cells due to any cause could have a significant effect on the results.

Quantitative PCR

$qPCR$ Specificity

Quantitative PCR is an immensely powerful and specific technique. Theoretically, this assay can accurately detect minute variations in target sequence copy number – even between samples containing only 10 and 5 copies of a primer-specific sequence. However, equally important is the immense significance we are ascribing to this variation, as well as the assumptions we are making when using VP1 as a proxy for BK viral replication.

Our approach was to examine BK VP1, a gene transcribed and translated late in the BK replication cycle, presumably produced only after the BK viral genome has reached the nucleus and has successfully expressed early genes such as Large T antigen. Upon observing lower levels of transcript for VP-1, we concluded that BEZ-235 treatment had led to less efficient BK viral replication. Further studies are needed to validate the appropriateness of VP1 as a marker of replicative efficiency. What about the VP1 amplified from virion capsid coats that were not able to initiate a productive infection? What about those virions still contained in acidified vesicles, or in the process of engaging ERAD pathway machinery? An early step in the DNA extraction process is
cell lysis. This includes degradation of any and all cellular plasma membrane components – allowing for the pooling of productively and non-productively-infecting BK virions from nuclear, endosomal, and ER-associated compartments. If the experimental question to be addressed is what the effects of BEZ-235 are on production of infectious BK virions, these issues represent a potential source of error in the interpretation of qPCR results.

Precisely because of the ability of qPCR to detect immensely small differences in copy number, human errors that introduce ‘massive’ variation in copy number become increasingly significant. Practically, what this means that the results produced during PCR analysis are just as likely due to ‘experimental error’ and accidental contamination, as they are to the intracellular effects of BEZ-235 on viral replicative efficiency.

*Experimental Errors*

Experimental error in qPCR assays comes from many sources. One major source may have had to do with the viral infection protocol, which is common to all three of types of assays in this project. After counting a well to determine the correct number of BK virions to add to each well for an MOI of 1.0, this number of virions was then added to each of five separate glass tubes (one for each experimental well) containing 1 mL of media each (just enough liquid to cover the slide bottom, thus facilitating infection). After brief vortexing, the homogenized viral suspension was then pipetted into the wells. The original
reasoning was that having eight individual viral suspensions would allow for more accurate virion distribution to each of the wells. However, this was not the case.

Unnecessary pipetting, especially of concentrated solutions, represents a potential source of error. Given that BKV was stored in aliquots of $2.5 \times 10^6$ virions per 54.9 µL (~45,537 virions per µL), the pipetting of even a single µL of suspension represents a huge number of infectious units in highly concentrated form. After accounting for the fact that a 10 µL pipette holding 2.31 µL of liquid might reasonably contain 2.29, or 2.34 µL of volume, it becomes easy to see how repeated pipetting steps could lead to increased variation in delivery volume. Pipettes are recalibrated each year for exactly this reason, but it is not unreasonable to assume some variation is unavoidable. This variation may have resulted in a broad range in the number of infectious units delivered to experimental samples, and consequently a range of infectious events that took place over the course of the experiment.

Other error sources include: a) incomplete cellular homogenization or incubation with lysis buffer, such that not all genetic material was extracted from other cellular components (membranes, organelles, etc). CCD-1105 cells incubated at higher concentrations of BEZ-235 may be more susceptible to the lytic effects of the reagents contained in the lysis buffer. Another error source may have been the loss of cells via mechanical suction during any one of many aspiration steps (removal of media containing BEZ-235, removal of PBS washes, etc).

Loss of cells via aspiration may partially explain the trend seen in the results seen in Figure 15. If we assume that increasing concentrations of BEZ-235 cause decreased
cell viability, via the decreased translation of attachment or adherence proteins, it is possible that the cell population incubated with 1000nM BEZ-235 could be relatively more sensitive to aspiration than cells incubated with lower concentrations. This may have resulted in a decrease in overall DNA content recovered from this treatment condition (a ‘decrease in the denominator’). The result might be a higher ratio of copies BKV/µg DNA, when perhaps the actual trend, given a constant mass of genetic material, is one of ‘constancy’ across all drug treatments.

Finally these cell populations may have simply been overloaded with too much BK virus, thereby reducing any observable down-regulation of viral replication induced by BEZ-235. This is especially likely given the indirect mechanism of action of this drug in affecting BK viral replication. If we assume that BEZ-235 does appreciably down-regulate mTOR pathway activity and results in decreased overall ribosomal biogenesis, just how much of an effect is this likely to have? What degree of decrease (in rate of ribosomal biogenesis, or percent reduction in ribosome number) is required to appreciably affect the rate of viral replication? Perhaps a reduction of the MOI from 1.0 to .1, or .01, along with a prolonged BEZ-235 incubation time and more insight specifically into levels of ribosomes themselves, might allow us to further tease out answers to these questions.
Potential Further Directions in qPCR Experimental Design

As I realized only late in my project, the use of an internal qPCR control may have been helpful in discerning whether or not equal amounts of sample had been loaded into all reaction wells. An ‘internal’ control in this instance would involve adding a set of primers and probe specific for a host cell genomic DNA sequence, such as the genes encoding the cytoskeletal proteins actin or tubulin, or many other possible DNA sequences, whether they be within genes or non-transcribed regions of the human genome. Such an analysis would control for the effects of BEZ-235 on host cell proliferation or survival. An internal qPCR control for a host cell gene may have proven to be a more accurate way of insuring that we were actually comparing the number of BK virion genomes from equivalent numbers of host cells.

Amplification of this gene would occur simultaneously with that of BK VP1, in a single PCR reaction well. Although separate standard curves would need to be generated and run for each gene amplification, it is likely that the inclusion of an internal control could provide an increase in experimental accuracy and scientific confidence in the PCR results generated. A second amplification curve, unaffected by the experimental condition, would allow for monitoring of inconsistent sample loading as a source of error. This would allow for more accurate determination of the effect of BEZ-235 on BK viral replicative efficiency.
Western Blot Assays

Using Western blot assay were used to determine the activity levels of mTOR proteins at various levels of the pathway, notably by assaying mTOR and its upstream regulator Akt. Importantly, these proteins exist in roughly constant quantities in the intracellular environment; it is their ‘activation state’ (i.e. phosphorylation at particular known residues), rather than modification of the number of molecules, that up- or down-regulates activity of the pathway as a whole. After all, signaling to increase transcription and translation of any protein may represent a large period of time on a cellular scale. This may have implications for the duration of BEZ-235 treatment in order to induce a decrease in the rate of ribosomal biogenesis. Further investigation of this phenomenon is needed.

Western blotting represents the major investment of time and energy in this thesis project. Many electrophoresis and blotting assays (~9 total) were carried out, using samples from independent experimental time-courses representing ~150 hours of cell growth. Only a small number of these assays were of sufficient quality to be presented here. This is largely due to the significant number of optimizations and small adjustments that must be performed in order to produce a ‘clean’ blot – one that shows experimental information that is both clear (i.e. interpretable) and that investigates targets of interest.

From the outset, this project had three separate but related goals for the use of Western blot assays in the experimental BKV infection time-course described above (Figure 14). The first was a proof of concept; that BEZ-235 actually does, in fact, down-
regulate the mTOR pathway specifically and potently in non-virally infected cells, as it has been shown to do by previous studies. Secondly, we aimed to show that BEZ-235 induces a dose-dependent down-regulation of mTOR pathway intermediates in BK virally infected cells. Third, we sought to show that treatment with BEZ-235 reduced the replicative efficiency of BKV. ‘Replicative efficiency’ is not an easily defined viral attribute; thus we used the expression of a key BKV replicative protein expressed early in the replicative cycle, BK Large T antigen, as a proxy marker.

The results of Western blot experiments produced in this thesis project have answered some of these research questions to a greater degree of certainty than others. Staining for Akt Serine-473 phosphorylation in non-virally infected cells produced a dose-dependent decrease in band intensity that was reasonably strong, clear, and reliable (Figure 18). This is important because BEZ-235 does not directly inhibit Akt-phosphorylation at this site; rather it inhibits PI3K activity, which mediates Akt phosphorylation through secondary signaling mechanisms (i.e. PIP_3 and PDK-1). Perhaps the same is true for its direct inhibitory effect on mTOR (which we demonstrated weakly in Figure 20), and downstream effect on P70S6K (whose activity was not tested in this thesis project).

Perhaps most importantly, we showed a fairly clear decrease in BK Large T antigen band intensity with increasing concentration of BEZ-235 (Figure 19). This result is exciting; it supports the hypothesis that BEZ-235 inhibits or slows the rate of BK viral replication. However, it is also possible that Large T antigen band intensity was due to the fact that fewer CCD-1105 cells were present; i.e. as an artefact of the drug’s
cytostatic effect on cell growth. The pan-Akt loading control should show even amounts of cellular lysate loaded per well. In the SV40 Large T staining experiment, however, bands appeared to stain strongly – making analysis difficult.

Potential Further Directions in Western Blot Experimental Design

We did not have sufficient time to test for levels of all significant mTOR pathway proteins, or begin to determine relationships in the setting of viral infection (for example, investigating the extent of positive or negative mTOR feedback activity in virally infected vs. non-infected cells). In particular, no assays were conducted looking at the downstream effectors of the mTOR pathway, 4EBP and P70S6K. The latter is the most direct regulator of the rate of ribosomal biogenesis and turnover in host cells, and thus needs to be investigated in the future.

Immunohistochemistry Assays

While the trend in Large T expression seen in Figure 20 may seem visually appealing and indicative of a strong dose-dependent effect of BEZ-235 on Large T antigen expression, drawing conclusions from this visual evidence alone can be misleading. One important factor that was taken into account was the total number of CCD-1105 cells present in each field of view. It can be reasonably assumed that, all other
conditions held equal, fields with higher total number of CCD-1105 cells will also have higher counts of BKV-infected, large T antigen-expressing cells.

The total number of DAPI-stained cell nuclei per field were analyzed using the freeware program ImageJ. Earlier in the project, analysis of Large T antigen and DAPI expression was attempted with the freeware program CellProfiler (Broad Institute). This analysis stalled and was eventually discarded for use of ImageJ, when the CellProfiler program began labeling single nuclei as multiple objects. This led to vast discrepancies in cell counts, and an unacceptable level of inaccuracy in the total object counts that this program was reporting. Thus, analysis with CellProfiler was discontinued, in favor of ImageJ.

Final analysis with ImageJ showed DAPI-positive point maxima cell counts (found in Table 3) that substantially decrease as BEZ-235 concentration is increased, particularly at 1000nM BEZ-235. This may be indicative of BEZ-235 mediated reduction in cell growth; the drug may be affecting the length of time cells must remain in G1 phase of the cell cycle in order to fully replicate all necessary host cell proteins before progressing to mitosis and cell division. By slowing the overall rate of host cell replication, BEZ-235 may have, after 48 hours of treatment, reduced the total number of cells available to be stained for DAPI. Further experiments are needed to substantiate this claim. One approach would be to examine the effect of BEZ-235 on critical cell cycle proteins such as cyclins or cyclin-dependent kinases (CDKs).

Given that IC_{50} values for enzymes affected by this inhibitor are in the sub-50 nM range (see above), using this concentration for any assays (which represents more than...
20x the useful range of this drug) is not a useful measure of physiologic conditions in a human kidney.

*Sampling Bias in Immunohistochemical Imaging*

One major source of error in producing the cell counts for Large T and DAPI-stained cells is sampling bias, which concerns systematic bias incurred during the process of images were captured. Images were selected ‘randomly’ for further analysis while scanning around the microscope. This randomization process was accomplished by brief ‘blind rotation’ of the axes that held the slide in place: without looking into the microscope itself, or viewing the image as it appeared on the computer screen. Identical DAPI/Large T antigen screen captures were then taken at that location.

A significant assumption involved in this method was that the averaged positive counts from these five images are representative of cell distributions across the entire chamber slide. The growing surface of each slide slide was relatively large (4cm²); thus five images per slide, at 200x magnification, cover only a tiny fraction of its total surface area. Large deviations from the ‘true’ average values, in even one of the five images, can significantly affect the averaged results, and the conclusions that can be drawn from them. It is likely that taking images at a lower magnification, covering a larger surface area and with more positive countable events per image field, could reduce the effects of sampling bias.
Concluding Remarks

Collectively, the results of these assays have shown that there is promise in using BEZ-235 as a targeted antiviral agent against BK virus. As conclusive and reproducible qPCR analysis showing clear dose-dependent trends was lacking in this project, PCR data showing definitive reduction in viral genome number would be an important first step in further drug validation. Further testing and experimentation is needed to show the degree and sensitivity of BEZ-235 within the complete scope of human physiology, and how this drug affects viral replication itself, not just the proxy markers Large-T antigen or VP1. The development of an animal model of BK infection would greatly facilitate the discovery of answers to these questions.

Regardless of whether or not BEZ-235 consistently and in actuality reduces viral replication in a cellular model of BKV infection, it is an entirely different question to ask whether it does so in an intact human kidney experiencing BK viral reactivation. Hopefully, a more systematic approach by researchers in the coming months or years will yield data convincing enough to see BEZ-235 through to clinical trials as a BK-specific peri-transplant antiviral agent.
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EDUCATION

Boston University School of Medicine, Boston, MA
Masters in Medical Sciences, May 2014 (expected) GPA (to date): 3.79

Colby College, Waterville, ME
Bachelor of Arts, May 2012
Major: Biology, with pre-medical concentration GPA: 3.33
Minor: Geology

WORK EXPERIENCE

Beth Israel Deaconess Medical Center, Center for Virology and Vaccine Research, Research Student Boston, MA June, 2013 to present
• Tested efficacy of novel antiviral agent against BK viral growth in kidney cell lines, via cytotoxicity assays, dose-response studies, Western blots, quantitative PCR, and Immunohisto-chemistry (IHC) assays.
• Learned cell culture techniques, including cell passaging, peripheral blood mononuclear cell isolation, and the use of appropriate personal protective equipment and sterile procedures.
• Attended weekly lab meetings, participated in the discussion of current BK virus literature, and gave presentations on study design and review of current literature.

Boston University School of Medicine, Boston, MA October, 2013 to present
Physiology Tutor
• Instructed one student in Medical Physiology for two hours each week. Discussed ways to master the course material, provided guidance on study techniques and strategies, and reviewed quiz and test questions.

Clover Food Labs, Cambridge, MA September, 2013 to present
Team Member 12 hrs/ week
• Prepared sandwiches, platters, salads, and drinks for customers. Also worked as a fry cook, preparing falafel, eggplant, rosemary fries, and other items.
• Restocked walk-in freezer with foodstuffs, washed dishes, and cleaned friers.
• Instructed other team members in appropriate food preparation and cleaning protocols, and interacted with customers in a cheerful and positive manner.

Maine General Medical Center, Waterville, ME January, 2012
Intern
• Worked with five physicians (all hospitalists) on medical floor rounds, over January Term; was able to compare/contrast doctor-patient conversational style, doctor and support staff interactions.
• Discussed and observed use of medical terminology and electronic medical record keeping systems, familiarized self with patient charts, PET, MRI, EKG, and ultrasound results.

Colby College, Department of Biology, Waterville, ME  Fall, 2010 to Spring, 2012
Biology Tutor
• Provided individual instruction to multiple students with Introductory Biology coursework, 1-2 hours/week.

Colby College Department of Biology, Waterville, ME  Spring and Summer, 2011
Research Assistant
• Measured protein levels via gel electrophoresis in Drosophila melanogaster flies in the laboratory of Professor Tariq Ahmad.
• Carried out larval brain dissections in preparation for neuronal cell culturing.
• Quantified larval responses to olfactory and phototactic cues, via tracing and spatial analysis.

Assistant Trip Leader
• Conducted logistical planning for two to four week wilderness canoe trips for adolescent campers.
• Led workshops facilitating team building and gear and paddling safety.
• Led whitewater canoe travel on multi-week trips in Northern Maine, Quebec, and New Brunswick, with camper groups of six to fourteen.

VOLUNTEER EXPERIENCE
bWell Center, Pediatrics Department, Boston Medical Center, Boston, MA  Fall, 2013 to present
Volunteer
• Provided advice to young patients and their families as they entered the Pediatrics Department; including directions to departments in the hospital, signing up for various programs, checking out library books from our ‘satellite’ branch at the bWell center,
• Assisted in implementing the ‘jump-rope clinic,’ helping patients achieve realistic personal fitness goals (# of jumps in 30 seconds, # of jumps in 60 seconds, etc).
• Provided personalized health and wellness information to parents based on their specific needs, after proactively initiating conversations.

Greater Boston Food Bank, Boston, MA  Spring, 2013
Volunteer
• Assisted Food Bank employees and other volunteers in helping food distribution agencies load their bulk food orders. Gained proficiency in hand forklift usage.

Evening Sandwich Program, Universalist-Unitarian Church, Waterville, ME  Fall 2008 to Spring, 2012
Program Leader
• Coordinated weekly carpooling to and from the volunteer site through extensive email communication with student volunteers and the volunteer site coordinator.

• Cook and serve sandwiches and soups for low-income residents of Waterville, Skowhegan and Oakland; 6 hours/week.

• Met with other program leaders monthly; generated ideas for sustained campus activism and increased off-campus volunteering presence.

• ESP work was highlighted in a Colby newspaper article and appreciation banquet, December, 2011.

Inland Hospital, Waterville, ME
Volunteer

• Worked in Post-Operative Surgical Services: cleaned rooms and made patient beds, assisted patients with wheelchair transport, and interacted with RNs and doctors in the ward (Spring, 2012).

• Worked as a greeter at the front desk, signed patients in for registration, directed patients to other areas of the hospital, assisted patients with wheelchair transport. (Fall, 2011).

SPORTS AND CLUBS
Woodsman Team, Colby College
Fall, 2008 to Spring, 2012

• Engaged in competitive lumberjacking activities in an intercollegiate league, including chopping, pole climb, log decking, birling, and fire-build events. Practice was held 4x a week.

• Certified in chainsaw safety, cold-weather start, chain sharpening, etc.

Gentleman of Quality Club, Colby College
Fall 2010 to Spring, 2012
Volunteer Coordinator

• Encouraged volunteering amongst members, in ESP as well as other volunteer programs. This encouragement directly resulted in several members becoming regular volunteers.

• Generated ideas and publicity for club events such as a ‘GQ Real Talk’ during Fall, 2011, to which over 50 people attended.

CERTIFICATIONS AND RECOGNITION
Wilderness First Responder (includes CPR and Wilderness First Aid), May, 2011
Open Water SCUBA Diver, certified by PADI, December, 2010
Lifeguard Certification, December, 2009 and June, 2012
Colby Outing Club Photo Contest, First Place - Landscape Spring, 2008
Colby Off-Campus Photo Contest, Honorable Mention (SCUBA) Spring, 2012
BUSM Art Days, submitted ‘Morning Fog on the Allagash’ for exhibition, April, 2013

HOBBIES AND INTERESTS
Photography, Road Biking, Swimming, Canoeing, Personal Fitness, Climbing Trees, Baking Cookies and Breads.