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In vitro anti-BK polyomavirus activity of imidazo[1,2-*c*]pyrimidine and pyrimido[1,6-*a*]pyrimidine derivatives

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Five imidazo[1,2-*c*]pyrimidine and pyrimido[1,6-*a*]pyrimidine derivatives were designed, synthesized and evaluated for their antiproliferative activity and cytotoxicity. Primary bioassays in vitro showed that two of five synthesized compounds, 6-benzyl-8-(methylsulfonyl)-2,6-dihydroimidazo[1,2-*c*]pyrimidin-5(3*H*)-one, and 9-(methylsulfonyl)-7-propyl-2,3,4,7-tetrahydro-6*H*-pyrimido[1,6-*a*]pyrimidin-6-one possessed potent antiviral activity against BKV (EC_{50}: 0.66 μM and 0.96, respectively). The selectivity index of these compounds is similar to that of cidofovir. Although antiviral activity was evident in secondary assays, significant virus inhibition occurred at or near the cytotoxic concentration (SI_{90}=1). Here we show that substituted pyrimidine derivatives are a promising structure class of chemical compounds for the development of antiviral drugs against BKV infections. Hence these compounds may be taken as lead compounds for further development of novel antimicrobial and anticancer agents.

1. Introduction

Viruses are the leading cause of severe complications in immunocompromised patients. Human Polyomavirus 1 (HPyV-1), more commonly known as BK virus (BKV) or BK polyomavirus (BKPyV), is a common infection acquired in childhood which then becomes latent. Modern immunosuppressive therapy has dramatically improved graft survival in transplant patients. Transplant patients are prone to the reactivation of viruses which are usually latent in immunocompetent people, such as herpes viruses (CMV, EBV, HSV, VZV, and HHV-8) and polyomaviruses (BK and JC), whether those viruses are latent in the recipient or in the organ transplanted.[1] In particular, with the implementation of more potent immunosuppressive drugs, BKV has become an emerging pathogen in kidney and bone marrow transplant recipients where it often causes associated nephropathy and haemorrhagic cystitis, respectively. Among the various pathogens, BKV can directly cause graft dysfunction. BKV is a frequently reactivating in transplant recipients after kidney and haematopoietic stem cell

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transplantation.[2,3] This reactivation can lead to BKV-associated nephropathy and compromised kidney function.[4] Polyomavirus BK infection is asymptomatic but graft outcome is poor if the patient develops tissue-invasive nephropathy.

The basic treatment strategy is immunosuppressive drug tapering, which is currently the recommended treatment of BKV viremia after kidney transplantation,[5] and using immunosuppressive drugs with antiviral properties are under evaluation.[6] The inhibitors of the mammalian target rapamycin (mTOR) also have data supporting their potential use for BKV infections after transplantation.[7,8] Despite almost 50 years of research on BKPyV, there is still no effective antiviral therapy. There are few treatments and vaccines available for antiviral therapies thus far. However, vaccines are likely to suffer from waning $CD4^+$ T cell counts and thus antiviral therapies are a promising therapeutic strategy. Limited therapies exist to treat these normally benign infections, and with increasing numbers of patients displaying symptoms of immunodeficiency, the need for novel strategies by which to treat opportunistic viral infections is high.

Anti-BKV agents reported in literature include fluoroquinolones (ciprofloxacin, levofloxacin), nucleotide analogues (cidofovir, brincidofovir), and artesunate.[9,10] Fluoroquinolones, which inhibit Type II topoisomerases by stabilizing the linkage between the enzyme and DNA, have been shown to inhibit the BK virus replication. An effective and safe antiviral should have a selectivity index (SI) of > 10, but these agents have an $SI < 4$. Therefore, they may not be efficacious in eradication of the virus *in vivo*.[11] Current findings from *in vivo* prospective studies do not support the use of quinolones to prevent posttransplant BK virus infection in kidney-transplant patients receiving heavy immunosuppression.[12-14] Hence, fluoroquinolones are not recommended for prophylaxis or therapy.[15] Although the efficacy of quinolones is less than adequate for an anti-polyomavirus agent, these data suggest that the BKV encoded helicase activity may represent a significant drug target for further development.

Nevertheless, the cytosine analog, cidofovir, and its prodrug, brincidofovir (CMX-001), showed a significant inhibition of BK virus DNA replication *in vitro*. This has been used by clinicians both intravenously and as bladder instillation. However, there is only weak evidence for the use of cidofovir as an *in vivo* anti-BKV drug.[16] Also, the pronounced nephrotoxicity limits its use particularly in renal transplantation.[10] In addition, cidofovir-resistance has been noted for multiple viruses.[17] Cidofovir-resistance represents a significant limitation for use of this compound to treat polyomavirus replication.

Leflunomide and its active metabolite teriflunomide also have antiviral effect against BKV, which is thought to be due to inhibiting viral replication by disruption of virion assembly at the nucleocapsid.[18] This is accompanied by a significant host cytostatic effect with non-specific pyrimidine depletion.[19] Leflunomide activity against BKV is modest and the SI is low.[20] The *in vivo* data suggest that leflunomide may be a potentially effective medication for treating BKV without significant toxicity.[21] On other hand, pharmacodynamic analysis revealed no association between leflunomide concentrations and BK viral PCR reductions. Multivariate analysis demonstrated that leflunomide therapy was not associated with BK viral clearance.[22] In any event, randomized controlled studies are needed to determine the utility of leflunomide for BK viremia.

Currently, leflunomide, cidofovir, and quinolones are not Food and Drug Administration (FDA)-approved for BKV treatment. Tacrolimus, cyclosporine A and sirolimus are approved for the prevention of organ rejection in the kidney transplant recipients, but not specifically to BKV prevention or treatment. Individuals with weakened immune responses face the prospect of acquiring severe diseases from benign viral infections. Treating these diseases can be challenging due to impaired immune function. Thus, it is imperative to find novel antiviral therapies that target host or viral proteins and are able to work independently of the immune system. Thus, there is an unmet need for novel antiviral drugs.

The first therapeutic target is the viral receptor on host cells. Initial encounters between a virus and a host cell are mediated through viral surface components. The primary receptor binding determinant on the BKV capsid is the VP-1 protein. The virus attaches to the cell surface by recognition of oligosaccharides terminating in alpha(2,3)-linked sialic acid.[23] Anti-BKV activity of nine sialic acid derivatives have been tested.[24] Compound 2-(hydroxymethyl)-6-(nonyloxy)tetrahydro-2*H*-pyran-3,4,5-triol, showed antiviral activity with a EC_{50} of 6.35±2.17 μM, and a neutral red SI of 30.51±2.02. All other sialic acid derivatives tested were inactive.

Among compounds capable of disrupting viral transport a slight anti-BKV activity was found for chloroquine, nystatin, amphotericin B, and colchicine.[24] A recently identified inhibitor of viral transport, Retro-2cycl, and its derivatives have been evaluated to inhibit human BKV in tissue culture,[25] and in the kidneys of mice[26]. Variety of compounds targeting different steps in the BKV life cycle have been tested *in vitro*, however, none of these agents has shown efficacy in *vivo*.[26,27]

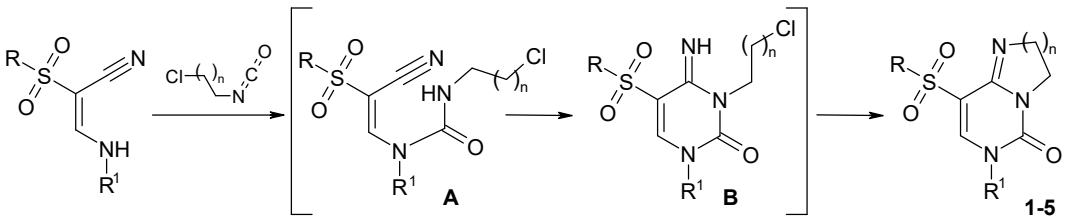
Unfortunately, no specific antiviral agent against BKV has been approved yet and the only therapeutic option is a modulation of the immunosuppressive drug regimen to improve immune control, though doing this may increase the risk of allograft rejection. Hence, there is an urgent need for development of a safe and efficacious antiviral agent against BKV.

Substituted pyrimidine derivatives (non-nucleosides) are found to be associated with various biological activities, including activity against different DNA and RNA viruses.[28] Previously, we showed that some pyrimidine derivatives have potent *in vitro* antiviral activity against polyovirus-3.[29] The present study focuses on the anti-BKV activity of pyrimidine derivatives which were synthesized at V.P. Kukhar Institute of Bioorganic Chemistry and Petrochemistry (Ukraine).

2. Results and Discussion

Chemistry

Imidazo[1,2-*c*]pyrimidine derivatives **1-3** and pyrimido[1,6-*a*]pyrimidine derivatives **4**,**5** (**Table 1**) have been synthesized from 3-(R-amino)-2-(methyl(phenyl)sulfonyl)acrylonitriles by refluxing with 1-chloro-2-isocyanatoethane or 1-chloro-3-isocyanatopropane and triethylamine[30] (**Scheme 1**).

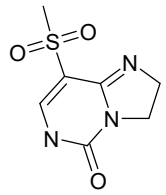
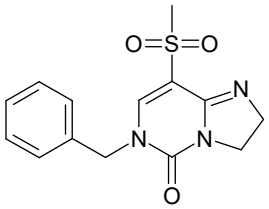
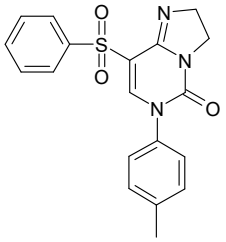
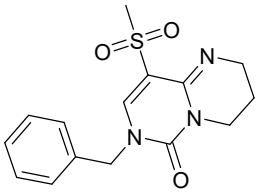
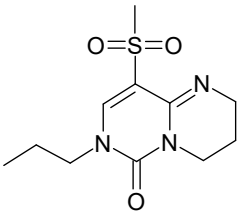


1 R=Me, R^1=H, n=1; **2** R=Me, R^1=Bn, n=1; **3** R=Ph, R^1=4-MeC_6H_4, n=1;
4 R=Me, R^1=Bn, n=2; **5** R=Me, R^1=Pr, n=2

Scheme 1. Synthesis of imidazo[1,2-*c*]pyrimidines **1-3** and pyrimido[1,6-*a*]pyrimidines **4,5**. Reagents and conditions: 1-chloro-2-isocyanatoethane or 1-chloro-3-isocyanatopropane, TEA, dioxane, reflux, 3h.

First, the isocyanate group acylates the NH fragment of the initial acrylonitriles to form intermediate **A**, followed by nucleophilic attack of the nitrile group, which leads to intermediate **B**. The intramolecular N-alkylation of the resulting imino group gives products **1-5**.

Table 1. Chemical structures of synthesized compounds

Compound	Structure	Name
1		8-(methylsulfonyl)-2,6-dihydroimidazo[1,2-*c*]pyrimidin-5(3*H*)-one
2		6-benzyl-8-(methylsulfonyl)-2,6-dihydroimidazo[1,2-*c*]pyrimidin-5(3*H*)-one
3		6-(4-methylphenyl)-8-(phenylsulfonyl)-2,6-dihydroimidazo[1,2-*c*]pyrimidin-5(3*H*)-one
4		7-benzyl-9-(methylsulfonyl)-2,3,4,7-tetrahydro-6*H*-pyrimido[1,6-*a*]pyrimidin-6-one
5		9-(methylsulfonyl)-7-propyl-2,3,4,7-tetrahydro-6*H*-pyrimido[1,6-*a*]pyrimidin-6-one

Biology

The obtained results of assays for BKV are summarized in **Table 2**. In general all the synthesized novel imidazo[1,2-*c*]pyrimidine and pyrimido[1,6-*a*]pyrimidine derivatives exerted antiviral activity *in vitro* against the BK polyomavirus strain. However, among the imidazo[1,2-*c*]pyrimidine and pyrimido[1,6-*a*]pyrimidine derivatives compound **2** and **5**, respectively, were the most effective ones in primary assays (**Table 2**). The compounds **1**, **3** and **4** showed insignificant activities against the BK polyomavirus with the range levels of SI_{50} from > 2 to > 7. The positive control compound cidofovir was active as expected in the assay. Although antiviral activity was evident in secondary assays, significant virus inhibition occurred at or near the cytotoxic concentration (SI_{90} = 1).

The nature of the substituent on the pyrimidine ring is determinant for the extent of each synthesized compound's activity, which might have an influence on its inhibiting mechanism of action. Attaching the phenyl group to the N-6 position in the pyrimidine ring of 8-(methylsulfonyl)-2,6-dihydroimidazo[1,2-*c*]pyrimidin-5(3*H*)-one (compound **1**) considerably enhances the antiviral activity of the latter (compound **2**), whereas a substitution of the methyl substituent by the phenyl one in the

sulfonyl group results in an inverse effect (compound **3**). On the contrary, the insertion of the phenyl substituent to the analogical position in the pyrimidine ring of the pyrimido[1,6-*a*]pyrimidine structure (compound **4**) reduces the antiviral activity relative to the similar imidazo[1,2-*c*]pyrimidine derivative (compound **2**). But on the other hand, the substitution of the phenyl group in this place by the alkyl group increases the antiviral activity (compound **5**). These observations may promote further developments of our research in this field to obtain compounds with a better pharmacological profile than standard drugs and serve as templates for the construction of better drugs to combat the viral infection.

Table 2. Antiviral activity and cytotoxicity of the pyrimidine derivatives against BK polyomavirus (strain Gardner) in HFF cell line. Compound concentrations are in μM.

Compound	strain Gardner				
	EC_{50}	EC_{90}	CC_{50}	SI_{50}	SI_{90}
			Primary assays*		
1	22.39	142.16	>150.0	>7	>1
2	0.66	1.03	>150.0	>229	>146
3	93.44	114.90	>150.0	>2	>1
4	21.62	28.33	>150.0	>7	>5
5	0.96	1.14	>150.0	>157	>132
Cidofovir	0.24	9.53	>150.0	>600	>16
			Secondary assays&		
2	31.1	>100.0	>100.0	>3	1
5	31.7	>100.0	>100.0	>3	1
Cidofovir	0.42	41.87	>100.0	>238	>2

* – Control and drug concentration ranges are 0.048-150 μM. & – Control and drug concentration ranges are 0.001-100 μM. Vehicle is DMSO. EC_{50} - compound concentration that reduces viral replication by 50%. EC_{90} - compound concentration that reduces viral replication by 90%. CC_{50} - compound concentration that reduces cell viability by 50%. SI_{50} - CC_{50}/EC_{50}. SI_{90} - CC_{50}/EC_{90}

The large T antigen (LTA) coding almost one half of the viral genetic information was evaluated as a potential target. It was reported that dihydropyrimidine derivatives inhibit BK polyomavirus replication and propagation by specifically inhibiting T antigen, and/or a cellular chaperone Hsp70.[31] It is possible that a similar mechanism could also turn out to be one of the ones intrinsic to the tested compounds in this report.

The synthesis of new molecules that parallel certain critical structural properties of compounds with known bioactivity is an important aspect of antiviral drug development. Additional research needs to be done to better understand BKV biology, which could give direction to the ongoing antiviral screening of lead compounds and could also enable the identification of potential new targets to block BKV replication.

3. Conclusions

Pyrimidine is a core structure in a wide variety of compounds that have diverse biological and chemical applications. Pyrimidine derivatives are therefore a potentially useful starting point in the drug discovery process. In this study, five substituted pyrimidine derivatives were synthesized and their antiviral activity against BKV was tested. Primary screening showed that two of the compounds exhibited favorable SI, however secondary testing of these two compounds demonstrated insignificant antiviral activity against BKV. While this study was unable to confirm the antiviral activity of this series of compounds, it does provide useful information on specific analogs with antiviral activity *in vitro* that can be further modified to identify more active inhibitors.

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4. Experimental

General procedure for the synthesis of compounds 1-5

To a solution of appropriate 3-(R-amino)-2-(methyl(phenyl)sulfonyl)acrylonitriles (2.15 mmol) in dioxane (5 ml), 0.27 g (2.26 mmol) of 1-chloro-2-isocyanatoethane or 0.31 g (2.26 mmol) of 1-chloro1-3-isocyanatopropane and 0.4 ml (2.29 mmol) Et3N are added successively. The reaction mixture is boiled under stirring for 2 h, then cooled to room temperature and the precipitate is filtered off with Et3N·HCl. The filtrate is evaporated under reduced pressure, the residue is recrystallized from ethanol. Physical and spectral properties of compounds **1-5** see in article.[30]

Cells culture and virus strains

The preparation of human foreskin fibroblast (HFF) cells was described by methods published previously and were derived from human foreskin tissue obtained from the University of Alabama at Birmingham tissue procurement facility with approval from the institutional review board.[32] The Gardner strain of BKV was obtained from ATCC (Manassas VA). HFF cells were passaged in minimum essential media (MEM) with Earle's salts with the addition of 10% fetal bovine serum (FBS, Hyclone, Inc. Logan UT), and standard concentrations of L-glutamine, penicillin and gentamycin. All the HFF cells used in the study were utilized with fewer than 10 passages.

Antiviral and Cytotoxicity Assays

Cell viability was assessed with the CellTiter-Glo Luminescent Cell Viability Assay (Promega) using manufacturer's protocol. Standard methods were used to calculate the 50% cytotoxic level CC_{50}.[33]

Primary assays for BKV were performed in 384-well plates containing monolayers of HFF cells.[32] Compound dilutions ranging from 0.048–150 μM were prepared in plates containing cells which were subsequently infected at an MOI of 0.001. After a 7 d incubation, total DNA was prepared and genome copy number was quantified by qPCR using the primers for the BKV VP1 gene, and the BKV probe. Plasmid pMP526 served as the DNA standard for quantification purposes.[34] Compounds were confirmed in a similar assay in 96-well plates according to established laboratory protocols with the compounds added 1h post-infection to identify compounds that inhibit early stages of replication including adsorption and entry. Genome copy number was determined by methods described above. For all assays, the concentration of compound that reduced virus titer by 50% (EC_{50}) was interpolated from the experimental data. Cytotoxicity was evaluated in a parallel plate with equivalent compound exposure, an equivalent number of cells. Cidofovir was selected as reference compounds on the basis of its reported antiviral activity. It was purchased from the University of Alabama Hospital Pharmacy.

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