Bisacodyl Limits Chikungunya Virus in Vitro and Is Broadly Antiviral

Natalie June Lomascolo

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LOYOLA UNIVERSITY CHICAGO

BISACODYL LIMITS CHIKUNGUNYA VIRUS IN VITRO
AND IS BROADLY ANTIVIRAL

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
MASTER OF SCIENCE

PROGRAM IN INFECTIOUS DISEASE AND IMMUNOLOGY RESEARCH INSTITUTE

BY
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CHICAGO, ILLINOIS
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<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% cytotoxic concentration</td>
<td>CC50</td>
</tr>
<tr>
<td>50% Inhibitory concentration</td>
<td>IC50</td>
</tr>
<tr>
<td>bis-(p-hydroxyphenyl)-pyridyl-2-methane</td>
<td>BHPM</td>
</tr>
<tr>
<td>Chikungunya virus</td>
<td>CHIKV</td>
</tr>
<tr>
<td>Coxsackie B3 virus</td>
<td>CVB3</td>
</tr>
<tr>
<td>Cyclic adenosine monophosphate</td>
<td>cAMP</td>
</tr>
<tr>
<td>Developmental Therapeutics Program</td>
<td>DTP</td>
</tr>
<tr>
<td>Dimethyl sulfoxide</td>
<td>DMSO</td>
</tr>
<tr>
<td>Dulbecco’s modified medium</td>
<td>DMEM</td>
</tr>
<tr>
<td>Fetal bovine serum</td>
<td>FBS</td>
</tr>
<tr>
<td>Human rhinovirus 2</td>
<td>HRV-2</td>
</tr>
<tr>
<td>Keystone virus</td>
<td>KEYV</td>
</tr>
<tr>
<td>La Crosse encephalitis virus</td>
<td>LACV</td>
</tr>
<tr>
<td>Mayaro virus</td>
<td>MAYV</td>
</tr>
<tr>
<td>Multiplicity of Infection</td>
<td>MOI</td>
</tr>
<tr>
<td>National Institutes of Health</td>
<td>NIH</td>
</tr>
<tr>
<td>Newborn calf serum</td>
<td>NBCS</td>
</tr>
<tr>
<td>Phosphate-buffered saline</td>
<td>PBS</td>
</tr>
<tr>
<td>Term</td>
<td>Abbreviation</td>
</tr>
<tr>
<td>-----------------------------------------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Plaque forming unit</td>
<td>PFU</td>
</tr>
<tr>
<td>Quantitative Real Time PCR</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>Sindbis virus</td>
<td>SINV</td>
</tr>
<tr>
<td>Tris-buffered saline with Tween</td>
<td>TBST</td>
</tr>
<tr>
<td>Vaccinia virus</td>
<td>VACV</td>
</tr>
<tr>
<td>Zika virus</td>
<td>ZIKV</td>
</tr>
</tbody>
</table>
Identifying novel antivirals requires significant time and resource investment, and the continuous threat of viruses to human health necessitates commitment to antiviral identification and development. Developing antivirals requires years of research and validation, and recent outbreaks have highlighted the need for preparedness in counteracting pandemics. One way to facilitate development is to repurpose molecules already used clinically. By screening such compounds, we can accelerate antiviral development. Here, we screened compounds from the National Institutes of Health’s Developmental Therapeutic Program for activity against chikungunya virus, an alphavirus that is responsible for a significant outbreak in the Americas in 2013. Using this library, we identified several compounds with known antiviral activity, as well as several novel antivirals. Given its favorable in vitro activity and well-described in vivo activity, as well as its broad availability, we focused on bisacodyl, a laxative used for the treatment of constipation, for follow-up studies. We find that bisacodyl inhibits chikungunya virus infection in a variety of cell types, over a range of concentrations, and over several rounds of replication. We find that bisacodyl does not disrupt chikungunya virus particles or interfere with their ability to attach to cells, but instead bisacodyl inhibits virus replication. Finally, we find that bisacodyl is broadly antiviral against a variety of RNA viruses, including enteroviruses, flaviviruses, bunyaviruses, and alphaviruses; however, it exhibited no activity against the DNA virus vaccinia virus.
Together, these data highlight the power of compound screening to identify novel antivirals and suggest that bisacodyl may hold promise as a broad-spectrum antiviral.
Alphavirus Overview

Alphaviruses are frequent human pathogens and have caused significant outbreaks. Alphaviruses make up the only genus of the Togaviridae family, comprised of small enveloped viruses containing single-stranded positive-sense RNA genomes. Alphavirus infections are spread through the aid of arthropod vectors such as *Aedes aegypti* and *Aedes albopictus*, and through this arthropod vector, alphaviruses rapidly spread. The mosquito species *Aedes aegypti* is found in sub-tropic locations that possess a warmer climate. The spread of this species has become easier due to the increased lack of clean water supply or waste management systems found in several areas of Europe and Africa (1). The species has been able to move across the world to the Americas by infesting cargo ships. This ability to spread has been a critical part of alphavirus transmission, particularly for the viruses chikungunya, dengue, yellow fever and Zika virus (1). On the other hand, the species *Aedes albopictus* is known to not only survive in sub-tropic regions, but also in regions with moderate temperatures. This adaptation plays a major role in disease transmission into different climates (2). Transmission of alphaviruses can occur by two cycles, either a zoonotic cycle or urban cycle. A zoonotic cycle of transmission involves viral spread between mosquito and rodents, primates, and birds. An urban cycle of transmission occurs between a mosquito
and human. An example of the alphavirus chikungunya virus (CHIKV) transmission is represented below in figure 1.

Figure 1. CHIKV Transmission Cycle. Arrows represent the direction of CHIKV transmission. The urban cycle represents the transmission between mosquito and human. The Zoonotic cycle represents the transmission between mosquito and rodent. Modified image from (8)

Introduction to Chikungunya Virus Structure, Replication, and Symptoms

The CHIKV genome is roughly 12 kb containing a 5’ cap and 3’ polyA tail (3). The genome is surrounded by capsid proteins to form a nucleocapsid (4), which is then enshrouded by a lipid envelope containing the structural proteins E1 and E2. Protein E1 and E2 are critical for CHIKV host cell invasion. Protein E1 contains a hydrophobic fusion loop necessary for membrane fusion and E2 is necessary for viral particle binding to host cells (4). The formation of these proteins on the CHIKV surface creates an icosahedral symmetry (4). This structure is depicted in figure 2.
Figure 2. CHIKV Structure and Genome Diagram. The diagram above represents the CHIKV genome. The genome consists of structural and non-structure proteins. The non-structural proteins function to synthesize RNA (nsP1/nsP2), as a helicase (nsP2), and as a polymerase (nsP4). The structure proteins function as capsid and glycoproteins. The viral image taken from (5) displays the icosahedral structure of CHIKV.

Once CHIKV has fused with a host cell, the virus will enter by clathrin-mediated endocytosis. Upon internalization into the host cell, the viral particle will be delivered to the early endosome (4). Viral genomes will be released allowing for viral mRNA to be translated. The non-structural proteins, nsP1-4, will be transcribed first to create a viral replication complex responsible for the synthesis of a (-) sense viral RNA serving as a template for further viral RNA replication and translation (6). Lastly, RNA packaging and nucleocapsid generation will occur and glycoproteins will be sent to the surface of the infected host cell. CHIKV packaged particles will bud on the surface of the host cell and be released as a mature virion (6). The CHIKV replication cycle is displayed in figure 3.
Figure 3. CHIKV Replication Cycle. The diagram above displays CHIKV infecting a host cell. First, CHIKV structural proteins will bind to the outside of a host cell and allow for fusion. CHIKV will be internalized and delivered to the early endosome. Viral genomes will be released allowing for mRNA translation. Viral replication complexes will then synthesize a (-) sense viral RNA strand that will function as a template. Viral RNA replication and translation will occur leading to CHIKV packaged particles that will bud on the surface of the host cell. Mature virions will then be released from the host cell.

Viral spread was best demonstrated by the rapid emergence of chikungunya virus (CHIKV) in the Americas in 2013 (7). Upon introduction and spread of CHIKV, the pathogen established itself and its geographical reach is unlikely to subside. CHIKV transmission remains prominent in warm and temperate regions such as northern Africa, southeastern Asia, and South America (8). The spread of the mosquito vector compounds virus control. CHIKV infection cannot be spread from human to human which can be advantageous when preventing further transmission (9). Clinically, CHIKV infections can vary from person to person. Most infected individuals experience symptoms of fever and light joint pain for up to 10 days (9). Others, usually older adults, experience severe joint
pain for years (9). CHIKV infection can be characterized as a musculoskeletal disease (10). Initially, CHIKV symptoms are acute and include fever, headache, and joint pain. Post-acute infection can develop into chronic disease, involving persistent muscle pain and joint swelling, giving rise to persistent arthralgia (11).

**Antiviral Development**

There is no vaccination or drug therapy available for CHIKV infection, and, thus, antiviral development is necessary to target CHIKV and quell future outbreaks. Rapid drug screens provide an efficient way to identify unknown compounds with potential antiviral activity and has been used successfully in the identification of several lead compounds, including for the alphavirus Venezuelan equine encephalitis virus (25). The development of direct-acting antivirals (DAA), or molecules specifically targeting the virus, has shown promise in anti-HCV and -HIV drug development, but is complicated by the rapid emergence of resistant viruses. In contrast, targeting the host cell to inhibit cellular pathways essential to virus infection shows significant promise (26, 27) in terms of preventing antiviral resistance and has the potential for broad-spectrum antiviral activity. However, few such compounds have been identified and successfully implemented as an antiviral therapy. To facilitate drug development (antivirals and otherwise) NIH has established a repository of chemical compounds, which we and others have used to screen for antiviral activity.

Developing antivirals requires significant research effort to identify potential targets or to identify lead compounds with promising antiviral activity. Thus, developing antivirals requires years of work prior to consideration of clinical trials and the potential for approval.
A strategy to overcome this significant limitation is to rapidly screen compounds that are approved for human use for novel antiviral activity (12–15). Such screens have shown promise for diverse viruses, including Ebola virus (16), Zika virus (17), Japanese encephalitis virus (18), and coronaviruses (19), including SARS-CoV-2 (20, 21). Outbreaks of viruses lead to a surge in research activity on these pathogens and has enhanced the development of specific antivirals. Recent progress with SARS-CoV-2 antivirals demonstrated the importance of antiviral development, as well as the limitations of drug screens (22, 23). Importantly, the development of drugs such as remdesivir (24) required years of prior research. Thus, antiviral development requires sustained research input to not only address current infections but also has the potential to combat future outbreaks.

**Aims and Hypothesis**

We screened compounds of known structure for antiviral activity against CHIKV. Bisacodyl, a well-studied diphenylmethane derivative, was identified as one of the top hits. Bisacodyl is a stimulant and oral laxative, used to treat patients with chronic constipation (28). The molecule targets the colon, and it hydrolyzes to produce bis-(p-hydroxyphenyl)-pyridyl-2-methane (BHPM) (29), which we find also has antiviral activity. Overall, we found that bisacodyl and BHPM exhibit significant antiviral activity *in vitro* in several cell types. These molecules inhibit viral genome replication to reduce viral titers.

In Aim 1, we developed a screen to identify compounds that showed antiviral activity against CHIKV through rapid drug screen. I hypothesized several drugs would show antiviral activity against CHIKV from the drug plates given to us from NIH.
from aim 1 identify several compounds that show potent activity against CHIKV infection \textit{in vitro}.

In Aim 2, we characterized the effects of bisacodyl, one of the top hits on the screen, on CHIKV. I hypothesized that bisacodyl and bisacodyl's active form, BHPM, can prevent CHIKV infection. Results from this aim shows that both bisacodyl and BHPM have potent antiviral activity against CHIKV. The data suggest certain steps of the CHIKV life cycle are being impacted.

In Aim 3, we determined if bisacodyl inhibits CHIKV replication. I hypothesized that replication is being impacted by bisacodyl and therefore, reducing viral titers. Results from this aim show that bisacodyl hinders CHIKV replication together with binding to prevent CHIKV infection.

Data produced from these aims show that bisacodyl and BHPM are both antiviral against CHIKV infection \textit{in vitro}. In addition, we have identified bisacodyl impacts two separate parts of the CHIKV life cycle, including viral binding and replication.
CHAPTER 2: MATERIALS AND METHODS

Cell Culture

Cells were incubated at 37°C containing 5% CO\textsubscript{2} in Dulbecco’s modified medium (DMEM; Life Technologies) containing bovine serum and penicillin-streptomycin. Vero and HeLa cells (BEI Resources) were supplemented with 10% newborn calf serum (NBCS; Thermo Fisher). Huh7, 293T, and BHK-21 cells, were supplemented with 10% fetal bovine serum (FBS; Thermo Fisher).

Drug Treatment

Standard treatment experiments were as followed, Huh7 cells were infected at a multiplicity of infection (MOI) of 0.1 PFU/cell, unless indicated differently, with CHIKV, HRV-2, CVB3, MAYV, SINV, KEYV, LACV, ZIKV, and VACV, then were simultaneously treated with bisacodyl (Cayman Chemical), Strychnine N-oxide (NIH DTP Compound), Benzipiperylon (NIH DTP Compound), Ribavirin (VWR), Naloxone (NIH DTP Compound), Danazol (NIH DTP Compound) dissolved in dimethyl sulfoxide (DMSO) or water. Cells were incubated at 37°C in 5% CO\textsubscript{2} for 24-48 h. Chemical structures were recreated using Adobe Illustrator (Adobe).

Rapid Screening of Antiviral Compounds

Huh7 cells were seeded on 96-well plates and treated with a 1:100 dilution of each compound from the NIH DTP compound plates 4 h prior to infection. Cells were infected at a MOI of 5 PFU/mL and incubated for 48 h at 37°C in 5% CO\textsubscript{2}. Medium was
removed, cells were fixed with 4% formalin, and living cells were stained using crystal violet solution (Sigma-Aldrich). Excess stain was removed in a mild bleach solution, and the cells were allowed to dry for 24 h. Crystal violet stain was resuspended in 10% acetic acid. The absorbance at 590 nm was detected using a BioTek Synergy H1 plate reader.

**Viability Assay**

Huh7 cells, unless otherwise noted, were seeded in a 96 well plate. 24 h later, cells were treated with increasing doses of bisacodyl or BHPM. After 24 h of treatment, 30 μL of CytoTox-Fluor Cytotoxicity Assay Reagent (Promega) were added to each well and incubated in the dark at RT for 45 min. Fluorescence of the plate was taken using a SpectraMax iD3 fluorometer (485nmEx/520nmEm).

**Infection and Enumeration of Viral Titers**

LACV (product no. NR-540; BEI Resources) and KEYV (strain B64-5587.05; product no. NR537; BEI Resources) were derived from Huh7 cells. ZIKV (strain MR766), VACV (WR strain; provided by Tom Gallagher), CHIKV (strain 181/25 from BEI Resources), SINV, and MAYV (43) were derived from Vero cells. CVB3 (Nancy strain) and HRV2 were derived from HeLa cells. Various drug amounts were maintained throughout the course of infection as noted. For infection unless otherwise noted, virus was diluted in serum-free DMEM. Viral inoculum was overlaid on cells for 30 min and the cells were washed with PBS before medium replenishment. Supernatant was collected at the times noted. For plaque assays, dilutions of cell supernatant were prepared in serum-free DMEM and used to inoculate a confluent monolayer of Vero
cells for 10 min at 37°C. Cells were overlaid with 0.1% agarose in DMEM containing 2% NBCS. CHIKV, MAYV, SINV, CVB3 and HRV2 samples were incubated for 2 days, LACV samples were incubated for 4 days, VACV samples were incubated for 24 hours, and KEYV and ZIKV samples were incubated for 5 days at 37°C. Following appropriate incubation, cells were fixed with 4% formalin and stained with crystal violet solution (10% crystal violet; Sigma-Aldrich). Plaques were enumerated and used to back-calculate the number of plaque-forming units (PFU) per milliliter.

Direct Incubation

Stock CHIKV was generated on Vero cells. Stock was combined with 10, 100, 300, and 600 µM bisacodyl and incubated at 37°C for 4 h. Virus was then directly quantified via plaque assay for both untreated and treated samples. In addition, this experiment was repeated with a single drug dose of bisacodyl over the course of 24 hours. Virus was quantified from both treated and untreated samples via plaque assay every 8 h.

Wash Away Assay

Huh7 cells were left untreated or treated with bisacodyl overnight. Bisacodyl-treated cells were subsequently washed with PBS and replenished with fresh media not containing drug. The plate was infected with CHIKV MOI 0.1. At 24 hpi, virus was quantified via plaque assay.

Immunofluorescence Imaging

Huh7 cells were seeded on coverslips and treated with 175 µM bisacodyl or untreated 24 h prior to CHIKV infection. Cells were infected with CHIKV MOI 0.1 for 24
h then fixed with 4% formalin. Cells were blocked using a 0.2% triton and 2% BSA in PBS at RT. Cells were washed using PBS and incubated with primary anti-dsRNA monoclonal antibody J2 (1:500; SigmaAldrich) for 1 h at RT. Cells were washed with PBS and incubated with secondary goat anti-mouse antibody (1:1000) for 1 h at RT. Cover slips were mounted onto microscope slides using mounting media containing DAPI (Biotium) for nuclei visualization. As control, uninfected samples were imaged. Samples were imaged with a Zeiss Axio Observer 7 with Lumencor Spectra X LED light system and a Hamamatsu Flash 4 camera with appropriate filters using Zen Blue software with a 40× objective.

**Western Blot**

Samples were collected using SDS buffer and run on 15% polyacrylamide gels. Gels were transferred in the Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were blocked using 5% BSA in 1x Tris-buffered saline with 0.2% Tween (TBST) and probed with primary CHIKV E2 (1:1000; BEI Resources) and actin (1:1000) antibody (ProteinTech). Membranes were washed in 1x TBST and placed in secondary anti-mouse IgG antibody (1:15000;SigmaAldrich) and incubated at room temperature for 1 h. Again, membranes were washed in 1x TBST and SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermofisher Scientific) was applied to membranes and developed on a Molecular Imager Bio-Rad Gel Doc XR+ Imaging System (Bio-Rad).

**RNA Extraction and qPCR**

Cells were collected using Trizol reagent (Zymo Research). RNA was purified, DNAse treated (Zymo Research), and used for cDNA synthesis with 5X All-In-One RT
PCR Master Mix (BioBasic). cDNA was analyzed by qPCR using CHIKV specific primers (44) control actin primers, and SYBR Green mastermix (ThermoFisher Scientific) using a QuantStudio Real-Time PCR System (ThermoFisher) and the ΔΔCT method. These values were then normalized to untreated controls to allow for direct comparison between samples.

**Replicon Analysis**

Huh7 CHIKV replicon cells (30) (provided by Drs. Maryam Ehteshami and Raymond Schinazi) were seeded in a 96-well plate. After 24 h, cells were treated with either bisacodyl or BHPM. 24 h post treatment cells were lysed for 20 minutes using passive lysis buffer. Well contents were transferred to a solid white plate and luminescence were measured using a microplate reader with the addition of Renilla substrate. This experiment was repeated to incorporate multiple time points up until 48 h after treatment with 175 µM bisacodyl. Luminescence was measured at the indicated times.

**Statistical Analysis**

Prism 9 (Graphpad) was utilized to generate graphs and perform statistical analysis. For all analyses, ANOVA and two-tailed Student’s t test was used to compare groups, unless otherwise noted with α = 0.05.
Table 1. Top Hits in Initial Antiviral Screen.

<table>
<thead>
<tr>
<th>DTP NSC No.</th>
<th>Primary screen value (Absorbance)</th>
<th>Antiviral name</th>
<th>Description</th>
<th>Prior antiviral activity against:</th>
</tr>
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<tbody>
<tr>
<td>24951</td>
<td>0.590</td>
<td>Strychnine N-oxide</td>
<td>Strychnine derived Strychnosnux-vomica metabolite</td>
<td>SARS-CoV-2(31)</td>
</tr>
<tr>
<td>73254</td>
<td>0.403</td>
<td>Benzipiperylon</td>
<td>Anti-inflammatory drug</td>
<td>None known.</td>
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<tr>
<td>70413</td>
<td>0.296</td>
<td>Naloxone</td>
<td>Opioid receptor antagonist; Reversal of opioid overdose respiratory depression</td>
<td>None known.</td>
</tr>
<tr>
<td>270916</td>
<td>0.280</td>
<td>Danazol</td>
<td>Attenuated androgen; Treatment of gynecologic and hematologic disorders</td>
<td>HTLV-1, HCV, HIV(32, 33)</td>
</tr>
<tr>
<td>755914</td>
<td>0.265</td>
<td>Bisacodyl</td>
<td>diphenylmethane derivative; Laxative</td>
<td>EBOV(16)</td>
</tr>
</tbody>
</table>

Table 2. IC50 Values Derived From Bisacodyl Treatment on Diverse Viruses.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Cell type</th>
<th>IC50 value (uM)</th>
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</thead>
<tbody>
<tr>
<td>CHIKV</td>
<td>Huh7</td>
<td>39.1</td>
</tr>
<tr>
<td></td>
<td>293T</td>
<td>68.4</td>
</tr>
<tr>
<td></td>
<td>Vero</td>
<td>89.2</td>
</tr>
<tr>
<td></td>
<td>HeLa</td>
<td>53.7</td>
</tr>
<tr>
<td></td>
<td>BHK-21</td>
<td>69.9</td>
</tr>
<tr>
<td></td>
<td>MEF</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>HFF</td>
<td>4.2</td>
</tr>
<tr>
<td>HRV-2</td>
<td>Huh7</td>
<td>13.2</td>
</tr>
<tr>
<td>CBV3</td>
<td>Huh7</td>
<td>68.3</td>
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<td>MAYV</td>
<td>Huh7</td>
<td>79.2</td>
</tr>
<tr>
<td>SINV</td>
<td>Huh7</td>
<td>119.2</td>
</tr>
<tr>
<td>KEYV</td>
<td>Huh7</td>
<td>36.9</td>
</tr>
<tr>
<td>LACV</td>
<td>Huh7</td>
<td>293.9</td>
</tr>
<tr>
<td>ZIKV</td>
<td>Huh7</td>
<td>23.6</td>
</tr>
<tr>
<td>VACV</td>
<td>Huh7</td>
<td>519.2</td>
</tr>
</tbody>
</table>
CHAPTER 3: RESULTS

Rapid screening of molecules for activity against CHIKV infection. Rapid screening of molecules for activity against CHIKV infection. We initiated a drug screen using a selection of compounds available from the NIH DTP. Previously, we screened compounds for activity against La Crosse virus (LACV) using a similar strategy to identify novel antivirals (23). In these assays (summarized in Figure 1A), we plated human Huh7 hepatocytes to confluency in 96-well plates. Four hours prior to infection, we treated with a 1/100 dilution of the compounds. We subsequently infected the cells at multiplicity of infection (MOI) 5 plaque-forming units (PFU) per cell with CHIKV, to ensure infection of all cells, and allowed infection to progress for 48 h, at which point, we stained the cells with crystal violet. As controls, we left wells untreated (CHIKV only) or untreated and uninfected. Additionally, we treated cells with ribavirin, a known antiviral with activity against CHIKV (24–26), as a control. In these assays, any compounds with cytotoxicity or compounds that are ineffective against CHIKV both generate a readout of a cleared well and reduced absorbance. However, potential antivirals that were not cytotoxic would stain with the crystal violet and yield a higher absorbance. Thus, our screen is specific for compounds that are both nontoxic and exhibit anti-CHIKV activity.
Figure 4. Antiviral Screen Design and Identification of Primary Screen Hits. (A) Schematic of drug screen. Huh7 cells were treated with antivirals 4 h prior to chikungunya virus (CHIKV) infection at an MOI of 5. At 48 hpi, cells were fixed with formalin, stained with crystal violet, and quantified by plate reader. (B) Raw crystal violet stain quantification. Each compound is represented by a single dot, and compounds of highest interest are labeled, along with our control antiviral, ribavirin. (C) Analyzed antiviral activity results compared to not treated (NT) controls to analyze the relative antiviral activity. A value of 1 represents a treatment that resulted in crystal violet staining equivalent to untreated cells. Top antiviral hits against CHIKV were used to treat Huh7 cells with increasing doses of (D) strychnine N-oxide, (E) benzipiperylon, (F) naloxone, (G) danazol, and (H) bisacodyl and infected with CHIKV at an MOI of 0.1. Viral titers were determined 24 hpi by plaque assay. Error bars represent one standard error of the mean (N≥3 for panels D-H). *p<0.05, **p<0.01, ***p<0.001 by two-tailed Student’s T-test.

We quantified this signal by resuspending stained cells in acetic acid and reading absorbance (Figure 1B). Readings were pooled for each compound and normalized to control values obtained from uninfected, untreated cells on each plate (Figure 1C). Values equivalent to uninfected, untreated cells were considered to have antiviral
activity and were selected for additional screening (Table 1). Satisfyingly, ribavirin exhibited significant antiviral activity and was the third highest hit in our screen.

With the top five hits in our screen, we performed secondary screening. In these assays, we treated cells with escalating doses of the compounds 4 h prior to infection with CHIKV at MOI 0.1 to allow for multiple rounds of viral replication. We measured viral titers 24 h later via plaque assay. We observed that each of the compounds exhibited antiviral activity, to varying degrees. Treatment with strychnine N-oxide did not result in reduced viral titers (Figure 1D); however, treatment with benzipiperylon, naloxone, danazol, and bisacodyl produced a significant decrease in viral titer (Figure 1E-H). Our fifth highest hit in our screen, bisacodyl was of particular interest because of its strong antiviral activity as well as its physiological properties, including its characterization as a constipation treatment and general tolerability to human consumption (22). We also observed dose-dependent reductions in CHIKV titers with bisacodyl treatment, providing for a wide range of concentrations with antiviral activity. For these reasons, we chose to focus on bisacodyl for further study.

**Bisacodyl exhibits antiviral activity with limited cytotoxicity.** To begin, we measured viral titers with a range of bisacodyl doses, up to 175 µM (depicted in Figure 2A). We seeded Huh7 cells and treated them with bisacodyl 24 h prior to infection. Cells were then infected at a MOI of 0.1 to allow for multiple rounds of replication. At 48 hpi virus was collected and quantified via plaque assay (Figure 2B). We found that titers were significantly decreased at concentrations above 50 µM when compared to
untreated control. From this data, we calculated the 50% inhibitory concentration (IC50) value to be 39.1 µM (Table 2). We noted no obvious effect on cell viability in tissue culture with bisacodyl treatment by cellular morphology under the microscope, so to confirm that our treatments were not negatively impacting cell health, we performed a viability assay (Figure 2C). We seeded a confluent 96-well plate and treated with increasing doses of bisacodyl, including doses that well surpass its significant antiviral producing phenotype. We observed cells lived beyond the IC50, as shown by our viability assay readout. Toxicity was observed above doses of 400 µM bisacodyl, and the 50% cytotoxic concentration [CC50] value was calculated to be 475.1 µM for a selectivity index (CC50/IC50) of 12.2, which gives us a window of opportunity to treat cells with bisacodyl without impacting viability.
Figure 5. Bisacodyl Exhibits Antiviral Activity with Limited Cytotoxicity. (A) Schematic of bisacodyl treatment experiments (B) Huh7 cells were treated with increasing doses of bisacodyl 24 h prior to CHIKV infection at MOI 0.1. CHIKV viral titers were quantified by plaque assay at 48 hpi. (C) Huh7 cells were treated with increasing doses of bisacodyl 24 h prior to analysis for viability. (D) Vero, (E) HeLa, (F) BHK-21, (G) 293T, (H) HFF, and (J) MEF cells were treated with increasing doses of bisacodyl as in (A) and subsequently infected. CHIKV titers were determined 48 hpi by plaque assay. (I) HFF and (K) MEF cells were treated with increasing doses of bisacodyl and viability measured. Error bars represent one standard error of the mean (N≥3). *p<0.05, **p<0.01, ***p<0.001 by two-tailed Student’s T-test.

To confirm this antiviral phenotype of bisacodyl using additional in vitro model systems, we measured viral titers after bisacodyl treatment in several cell types, including Vero, HeLa, BHK-21, 293T, HFF, and MEF (Figure 2D-H, J). Cells were treated with increasing amounts of bisacodyl 24 h prior to infection at MOI 0.1. At 48 hpi virus was collected and quantified via plaque assay. Similar to our experiments with Huh7 cells, all cell types showed a similar decrease in titer with bisacodyl treatment.
(see IC50 values in Table 2). As with Huh7 cells, we observed minimal effects on cellular viability in HFF and MEFs (Figure 2 H, J). Bisacodyl showed the best antiviral activity in Huh7 cells, which we continued using for additional experiments. Overall, these data suggest bisacodyl can act as an antiviral in several cell types with favorable antiviral properties.

**Bisacodyl is antiviral over several rounds of replication and limits viral RNA and protein accumulation.** To characterize the effect of bisacodyl over multiple rounds of replication, we measured viral titers over a timecourse. We seeded Huh7 cells and treated with 175 µM bisacodyl 24 h prior to infection and subsequently infected at MOI 0.1 to measure viral replication over several rounds of replication. Samples were collected at regular time points throughout infection and quantified by plaque assay (Figure 3A). We found that bisacodyl reduced titers below untreated samples after 16 hpi and that titers never recovered in bisacodyl-treated cells, suggesting that bisacodyl treatment remained antiviral over several rounds of virus replication.
We further analyzed cells for viral genomes to determine if bisacodyl was affecting viral RNA accumulation. We sampled bisacodyl-treated and CHIKV infected cells and collected cells for RNA purification 24 hpi. RNA was purified, reverse transcribed, and analyzed by qPCR using primers specific to CHIKV and normalized to cellular actin using the ΔΔCT method (Figure 3B). These values were then normalized to untreated control conditions, set to 1, to compare untreated to treated conditions. We observed that bisacodyl treatment up to 200 µM significantly reduced intracellular CHIKV genome accumulation, in a dose-dependent manner. To complement these data, we also measured viral genomes in cellular supernatant, representing released viral genomes. We collected infected cell supernatant, purified and reverse transcribed RNA and performed qPCR with CHIKV-specific primers (Figure 3C). Released viral genome quantity was similarly reduced, fitting with diminished titers with bisacodyl
treatment, up to 200 µM. To confirm that this reduction in viral genome accumulation similarly reduced viral protein accumulation, we measured viral E2 protein by western blot after treatment with escalating doses of bisacodyl (Figure 3D). We observed that bisacodyl significantly limited E2 accumulation in infected cells in a dose dependent manner, similar to reductions in viral titers and genome accumulation. Finally, we imaged infected cells via immunofluorescence, staining for dsRNA (Figure 3E). We found that bisacodyl treatment did not change the gross morphology of these replication complexes within infected cells under the conditions tested. Thus, overall bisacodyl appears to disrupt viral RNA and protein accumulation, but not the formation of replication complexes, suggesting that while these replication complexes can form, they are not producing viral genomes or the viral E2 structural protein.

**Bisacodyl inhibits CHIKV replication.** In all previous assays, we added bisacodyl to cells prior to infection, and the compound was present throughout infection (see schematic in Figure 4A). To identify the stage of virus replication sensitive to bisacodyl, we again treated Huh7 cells with 175 µM bisacodyl at distinct times before and after infection at MOI 5 to infect all cells. Titers were measured by plaque assay at 24 hpi. As expected, treatment of cells prior to infection led to a significant decrease in viral titers; however, viral titers were also reduced when bisacodyl was added up to 8 hpi. However, treatment after 8 hpi resulted in no significant change in viral titers (Figure 4B), suggesting that early events up to and including viral genome replication were affected by bisacodyl treatment. To determine if this antiviral effect of bisacodyl could be
reversed, we treated cells with bisacodyl and prior to infection, washed cells with PBS before replenishing the cells with complete media, lacking bisacodyl. When we replaced drug-free media, we observed a nearly full rescue of viral titers (Figure 4C), suggesting that bisacodyl's antiviral activity requires consistent treatment throughout infection.
Figure 7. Bisacodyl Inhibits CHIKV Replication. (A) Schematic of treatment of cells with bisacodyl relative to infection. (B) Huh7 cells were treated with 175 µM bisacodyl at distinct times before and after infection with CHIKV at MOI 5. Viral titers were determined at 24 hpi by plaque assay. (C) Huh7 cells were treated with 175 µM bisacodyl 24 h before being washed with PBS to remove bisacodyl and subsequently infected with CHIKV at MOI 0.1. Viral titers were determined at 24 hpi by plaque assay. (D) Stock CHIKV was treated with bisacodyl and incubated at 37°C at the indicated doses. Titers were determined by plaque assay after 4 h incubation. (E) Stock CHIKV was incubated with 175 µM bisacodyl at 37°C for the indicated amount of time prior to viral quantification by plaque assay. (F) Scheme of attachment assays. (G, H) CHIKV attachment assay was used to quantify CHIKV attachment with bisacodyl treatment. Cells were treated with (G) increasing doses of bisacodyl 24 h prior to a 5-minute or (H) 175 µM bisacodyl over a 30-minute time period of CHIKV infection on ice. Cells were subsequently washed to remove unbound virus and bisacodyl, and attached virus was allowed to form plaques. Plaques were subsequently quantified 48 h later by crystal violet staining. (I) Huh7 cells harboring a CHIKV replicon encoding Renilla luciferase were treated with increasing doses of bisacodyl for 24 h before analysis for luciferase activity. (J) Replicon-harboring Huh7 cells were treated with 175 µM bisacodyl for the indicated times and Renilla luciferase activity measured. Error bars represent one standard error of the mean (N≥3). *p<0.05, **p<0.01, ***p<0.001 by two-tailed Student's T-test.
Given the effect of bisacodyl on virus titers and genome accumulation, as well as its chemical structure, containing multiple phenol rings, we hypothesized that bisacodyl may be directly inactivating virus. To test this, we directly incubated CHIKV stock with increasing doses of bisacodyl for 4 h, at which point we directly titered the samples (Figure 4D). We observed no significant difference in viral titers, suggesting that bisacodyl was not directly impacting virus infectivity. To confirm, we incubated CHIKV stocks with bisacodyl over a timecourse, up to 24 h. In untreated samples, we saw a decay in viral titers, but this decay was indistinguishable when incubated with bisacodyl (Figure 4E), again suggesting that bisacodyl did not directly inactivate viral particles.

Observing no change in CHIKV infectivity when incubated with bisacodyl, we next tested whether bisacodyl could inhibit viral attachment. To this end, we performed a viral attachment assay27, in which we incubate virus on treated or untreated cells for five minutes prior to washing and removing unbound virus and allowing a plaque to form (see schematic in Figure 4F). Importantly, in these assays, bisacodyl is present only prior to and during viral attachment before it is washed away to allow for plaque formation. We observed that bisacodyl treatment only modestly reduced viral attachment (Figure 4G), and the differences were not statistically significant. When this attachment assay was repeated with 175 µM dose of bisacodyl over a 30-minute period, we observed a significant reduction in bound virus (Figure 4H). These data suggest that bisacodyl inhibits viral binding by approximately two-fold, which suggests that additional stages in infection may be impacted by bisacodyl.
We next considered whether bisacodyl affected viral replication. Using Huh7 cells harboring a CHIKV replicon, encoding Renilla luciferase as a readout (27, 28), we treated cells with increasing doses of bisacodyl and measured luciferase activity 24 h later. We observed a dose-dependent decrease in luciferase activity (Figure 4I), suggesting that bisacodyl was inhibiting CHIKV replication. We confirmed this phenotype by treating with bisacodyl over a 24 h period, observing a steady and significant decrease in luciferase activity with increasing treatment time (Figure 4J). Together, these data suggest that at least one of bisacodyl’s antiviral effects is on CHIKV replication.

**The bisacodyl derivative BHPM is antiviral.** Bisacodyl must be initially converted to the metabolite bis-(p-hydroxyphenyl)-pyridyl-2-methane (BHPM, Figure 5A), the active metabolite, also known as desacetylbisacodyl, to relieve constipation (29). We aimed to investigate if this compound is also responsible for inhibiting CHIKV replication. We treated cells with increasing doses of BHPM 24 h prior to infection, as with bisacodyl treatment. The cells were infected with CHIKV, and virus was quantified 24 hpi via plaque assay (Figure 5B). We found a similar response to bisacodyl treatment with BHPM treatment, with significantly reduced viral titers above 50 µM and an IC50 of 38.6 µM. To ensure the compound treatment was responsible for this phenotype we again, as described earlier, performed a viability assay on Huh7 cells using increasing doses of BHPM (Figure 5C). We discovered that BHPM, like bisacodyl, is not toxic to cells until far beyond the effective dosage, with a CC50 value of 464.9 µM.
and a SI of 12.0. These data suggest that BHPM inhibits CHIKV infection similar to bisacodyl, and to confirm that BHPM similarly impacted CHIKV replication, we treated Huh7 cells harboring the luciferase-containing CHIKV replicon and measured Renilla luciferase activity 24 h after treatment (Figure 5D). As with bisacodyl, we observed a significant reduction in CHIKV replication above 50 µM, again suggesting that bisacodyl and its active derivative BHPM inhibit CHIKV replication.

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Figure 8. Bisacodyl's Active Metabolite, BHPM, is Antiviral. (A) Structures of bisacodyl and desacetyl bisacodyl, BHPM. (B) Huh7 cells were treated with increasing doses of BHPM 24 h prior to infection with CHIKV at MOI 0.1 Viral titers were determined 24 hpi by plaque assay. (C) Huh7 cell viability with BHPM treatment was measured after 24 h treatment. (D) Huh7 cells harboring a CHIKV replicon encoding Renilla luciferase were treated with increasing doses of BHPM. Luciferase activity was subsequently measured 24 h later. Error bars represent one standard error of the mean (N≥3). *p<0.05, **p<0.01, ***p<0.001 by two-tailed Student’s T-test.

Bisacodyl exhibits broad-spectrum activity against RNA viruses. To investigate how other viruses respond to bisacodyl treatment, we treated Huh7 cells with bisacodyl 24 h prior to infection with the enteroviruses Human Rhinovirus 2 (HRV-2) and Coxsackievirus B3 (CVB3; Figure 6A), the alphaviruses Mayaro virus (MAYV)
and Sindbis virus (SINV; Figure 6B), the bunyaviruses keystone virus (KEYV) and La Crosse virus (LACV; Figure 6C), the flavivirus Zika virus (ZIKV; Figure 6D), and the DNA virus Vaccinia virus (VACV; Figure 6E). At 24 hpi, virus was quantified via plaque assay. Identical doses of bisacodyl were used during treatments. We observed significant antiviral activity with bisacodyl treatment for all RNA viruses (see IC50 values in Table 2). KEYV was particularly susceptible, and titers could not be detected above 50 µM. Interestingly, the DNA virus vaccinia virus was not susceptible to bisacodyl treatment. These data suggest that bisacodyl is broadly antiviral, though its activity is limited to a subset of viruses.

**Figure 9. Bisacodyl Exhibits Broad Activity Against RNA Viruses.** Huh7 cells were treated with increasing doses of bisacodyl prior to infection with (A) the enteroviruses Human Rhinovirus 2 (HRV2) and Coxsackievirus B3 (CVB3), (B) alphaviruses Mayaro virus (MAYV) and Sindbis virus (SINV) (C) bunyaviruses keystone virus (KEYV) and La Crosse virus (LACV), (D) flavivirus Zika virus (ZIKV), and (E) the poxvirus Vaccinia virus (VACV). Viral titers were quantified by plaque assay 24 hpi in all cases. Error bars represent one standard error of the mean (N≥3). *p<0.05, **p<0.01, ***p<0.001 by two-tailed Student’s T-test.
CHAPTER 4: DISCUSSION

Antiviral development requires significant investment in identification of lead compounds, and testing approved molecules can provide a “jumpstart” to antiviral development. While a wide variety of antiviral screens have identified FDA-approved molecules, a limited set of these molecules have been used successfully as antivirals (12). However, testing these approved molecules has several benefits in terms of well-studied pharmacokinetic data in human patients that could be a significant hurdle for antiviral development de novo. While certainly not the only approach for antiviral development, screening approved compounds for alternative use as an antiviral holds significant promise. Here, we identified several interesting compounds (Table 1) with antiviral activity. Many of these molecules had previously shown antiviral activity (30, 31), validating our screening method. Our molecule of choice for follow-up study, bisacodyl, is used primarily as a treatment for constipation and has been used both clinically and over the counter. No prior work has suggested that treatment with bisacodyl reduces virus infection to our knowledge, though it was identified in a screen for antivirals to treat Ebola virus infection (5). Additional hits within our screen included naloxone, commonly used as a treatment for opioid overdose. Again, no studies have shown antiviral activity for this molecule. Danazol is used in the treatment of endometriosis (32), among other indications. These compounds, along with bisacodyl, are orally bioavailable (33), which is also promising for potential antiviral activity. While
our screen identified a plethora of compounds with potential antiviral activity, follow-up study will be necessary to confirm these results.

Bisacodyl has several properties that make it a potential antiviral. First, it exhibits broad-spectrum activity in vitro, suggesting that it could be used in the treatment of diverse viruses. Additionally, the drug itself is relatively nontoxic, both in vitro in our studies and in humans when it is taken to treat constipation. Toxic doses can be reached in humans when consistently exceeding 30 mg for several weeks (34), which may limit its potential antiviral activity. The drug is orally bioavailable and can also be taken as a suppository. The oral route and action on the gut has important implications for antiviral development, especially for fecal-oral pathogens, like several enteroviruses (including Coxsackievirus B3, studied here). The impact on the intestines and its effect as a laxative may require additional consideration for in vivo treatment, as laxative treatment can have multiple systemic effects (35, 36) and the effect of this amalgamation on virus infection would require significantly more study.

The precise mechanism by which bisacodyl impacts virus infection is not clear, though we believe that the molecule interferes at the stage of viral genome replication, as evidenced by the effect of bisacodyl on CHIKV replicons. However, bisacodyl may impact several viral proteins, or their expression or localization. We observe a significant reduction in the accumulation of viral RNA and E2 structural protein, though we observe no obvious change in replication compartment formation. Thus, bisacodyl may specifically impact the function of these replication compartments rather than their formation itself. Further, we observe a four-fold reduction in released viral genomes, despite reductions in titer of greater than 100-fold. Thus, bisacodyl may impact the
formation of infectious virions. Importantly, bisacodyl may impact a cellular process that, in turn, impacts virus replication. Bisacodyl’s molecular mechanisms are not fully understood. At an organismal level, bisacodyl induces peristalsis (37) by stimulating enteric neurons. Bisacodyl’s impact on chloride (38) and calcium (39) ions may play a role in this stimulation, though it has not been formally shown. Within the context of our antiviral screens, bisacodyl’s antiviral activity could be a result of perturbation of ion signaling, though the impact of chloride and calcium signaling on a broad array of viruses is incompletely understood. Thus, further pharmacological characterization of bisacodyl’s molecular mechanism will be necessary to fully understand its antiviral activity.

The use of a laxative such as bisacodyl in the treatment of virus infection has several implications, both in the treatment of disease and in the potential molecular mechanisms involved in infection. As mentioned, bisacodyl has several impacts on the body, both at the molecular and organismal level. Because bisacodyl’s primary effects are seen within the alimentary canal, it may function well in the context of a virus infecting these tissues, such as enteroviruses. However, the systemic effect of bisacodyl may well impact the replication of non-enteric viruses, like CHIKV. Significant additional study, including animal studies, would illuminate the possibility of using bisacodyl to treat CHIKV infection. For instance, it is unclear if oral bisacodyl would reduce viral replication in a systemic or arthritis model of CHIKV infection. Further, treating virus-infected patients with a laxative like bisacodyl may exacerbate their condition. Regardless, understanding the effects of bisacodyl on CHIKV infection, as
well as other virus infection, has the potential to highlight additional cellular pathways or physiological processes that are critical to virus infection.

Our data suggest that bisacodyl exhibits broad-spectrum activity against a variety of RNA viruses, from alphaviruses to bunyaviruses to enteroviruses. This could suggest a conserved mechanism of action whereby bisacodyl affects a common target. However, it is also possible that bisacodyl is targeting a host pathway that these RNA viruses rely on. Bisacodyl, as a laxative, has a variety of functions within the cell and the body, including releasing intracellular calcium and chloride ions, manipulating aquaporins (40, 41), and altering cAMP levels within the cell (42), though its molecular target is not fully understood. Thus, our studies are limited by the current understanding of bisacodyl’s mechanism of action within the cell. Interestingly, we find that vaccinia virus (VACV) is insensitive to bisacodyl. VACV, a DNA virus that replicates within the cytoplasm, has several characteristics that distinguish it from the other viruses in our study, most prominently that it is a DNA virus. However, VACV is vastly different in its replication strategies, and untangling the precise mechanism whereby VACV is insensitive to bisacodyl is unclear at this point. However, these data suggest that despite broad antiviral activity, bisacodyl is not a global antiviral.
REFERENCES


VITA

The author, Natalie J. LoMascolo, was born in Maywood, Illinois, on February 24th, 1998, to Joe and Kim LoMascolo. She attended Loyola University Chicago in Chicago, Illinois, where she earned a Bachelor of Science in Biochemistry. She matriculated to Loyola University Chicago Stritch School of Medicine in July 2020, in the Infectious Disease and Immunology Research Institute MS program. She joined the lab of Dr. Bryan Mounce where she investigated antiviral compounds against CHIKV and CVB3 viral fidelity. After completion of her graduate studies, Natalie will continue to work under the supervision of Dr. Bryan Mounce while pursuing a PhD at Loyola University Chicago.