Antiviral Nucleoside Inhibitors of Leishmania RNA Virus 1: Discovery and Mechanism

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Antiviral Nucleoside Inhibitors of *Leishmania* RNA Virus 1: Discovery and Mechanism

By

John Isaac Robinson

A dissertation presented to
The Graduate School
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

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John I. Robinson

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ABSTRACT OF THE DISSERTATION

Antiviral Nucleoside Inhibitors of *Leishmania* RNA Virus 1: Discovery and Mechanism

By

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Doctor of Philosophy in Biology and Biomedical Sciences

Biochemistry

Washington University in St. Louis, 2017

Professor Stephen M. Beverley, Chair

Some *Leishmania* parasites in the *Viannia* sub-genus are persistently infected with *Leishmania* RNA virus 1 (LRV1), a single-segmented double-stranded RNA virus belonging to the family Totiviridae. Infected parasites cause greater pathology and reach higher populations in mouse models of *Leishmania* infection. In human disease, LRV1+ parasites are correlated with increased frequency of treatment failure and relapse. Efficient methods to detect LRV1 and eliminate it from parasites are required to better understand the role of LRV1 in *Leishmania* infection. We optimized multiple techniques to measure LRV1 levels in parasites, most notably using flow cytometry to measure the amount of viral capsid protein in individual parasites stained with an anti-capsid antibody. A slot-blot based assay allowed us to rapidly quantify the levels of viral capsid protein or viral dsRNA. Armed with these tools, we screened various known anti-viral compounds and nucleoside analogs for compounds that could inhibit LRV1. The two compounds we identified, 2′-C-methyl-adenosine (2CMA) and 7-deaza-2CMA (7d2CMA), can be utilized to efficiently cure parasites of LRV1 and generate matched
LRV1+/LRV1- strains for further study. These pairs allowed us to confirm the role of LRV1 in exacerbating disease.

We then set out to determine the mechanism of LRV1 inhibition by 2CMA. I showed that the active form of 2CMA is its triphosphate (2CMA-TP), and that it directly inhibits the viral RNA polymerase activity with IC50 values ranging from 130 to >600 µM depending on the type of virus particle (empty, ssRNA, or dsRNA-containing) and the RNA product formed. However, this inhibition required much greater 2CMA-TP concentrations than expected based on the EC50 of 2CMA against LRV1 replication in parasites (3 µM). *Leishmania* are known to accumulate significant amounts of other purine analogs, so I measured the concentration of 2CMA-TP in parasites treated with 10 µM 2CMA and found that they accumulated 410 µM 2CMA-TP. I then created a simulation of LRV1 inhibition and used it to show that this level of 2CMA-TP accumulation was sufficient to replicate the LRV1 inhibition we previously observed experimentally. Monitoring 2CMA-TP levels in parasites after 2CMA was removed from their medium showed that they retained significant amounts of 2CMA-TP even after 8 hours.

Furthermore, culturing LRV1+ parasites in a low concentration of 2CMA slowly generated a significant population of LRV1-low parasites. These results suggest that our existing LRV1 inhibitors may be sufficiently potent to inhibit LRV1 in mice infected with LRV1+ parasites and determine if this reduces or eliminates the increased pathogenicity associated with LRV1. The data summarized herein enables quantification of LRV1 and controlled study of its effects on both parasites themselves and the host immune system. The essential role of purine salvage for the potency of 2CMA highlights the importance of that pathway in drug design for *Leishmania* and suggests further methods for identifying LRV1 or *Leishmania* inhibitors.
Chapter 1: Introduction
Preface

The first draft of this chapter was written by JIR. Comments from SMB were incorporated into the final version, presented here.
1.1 – Leishmaniasis: A Classic Neglected Tropical Disease

*Leishmania* is a genus of single-celled eukaryotic parasites transmitted by multiple species of sand flies. (Weigle and Saravia 1996) Various species of *Leishmania* are endemic to a wide swath of the globe, from Central and South America to North Africa, the Middle East, and Central Asia. (Pigott et al. 2014) In humans, infection with *Leishmania* parasites causes one of roughly three types of disease: cutaneous, mucocutaneous, or visceral leishmaniasis. Most infections begin as cutaneous leishmaniasis (CL). CL manifests as a skin lesion centered on the point of infection and is often self-limiting. Mucocutaneous leishmaniasis (MCL) occurs when the parasites metastasize from the initial lesion to mucous membranes, often including the nasal passages. These tissues are progressively destroyed by high levels of parasite-induced inflammation, leading to disfiguration and significant social stigma. (Kassi et al. 2008) Visceral disease is characterized by parasite dissemination to the internal organs, where it is fatal if left untreated. (McGwire and Satoskar 2014) The factors determining disease progression are thought to be both host and pathogen derived. (Hartley et al. 2014; Kaye and Scott 2011)

The total number of people at risk of leishmaniasis is over 1 billion worldwide. (World Health Organization 2016) Recent estimates put the number of new cases per year at around 1.2 million, although the frequency of asymptomatic infections makes this number uncertain. (Alvar et al. 2012; den Boer et al. 2011) Poor reporting rates and the prevalence of asymptomatic or sub-clinical infections mean that the true infection rates are likely much higher. (Singh et al. 2006; Ostyn et al. 2011; Weigle et al. 1993) Although leishmaniasis is usually not considered endemic in the United States, recent cases of local transmission in Texas have been reported. (Clarke et al. 2013) Another local risk comes from foxhounds: the majority of American foxhounds have chronic *Leishmania* infections that are transmitted from mother to pup. (Petersen
and Barr 2009) This particular strain of *Leishmania* can be transmitted by sand flies, but no vector-borne transmissions have yet been reported, despite the presence of sand flies in the United States. (Schaut et al. 2015)

Currently-available treatments for leishmaniasis are somewhat limited. The most common drugs are pentavalent antimonials – antimony complexed with various organic compounds. These therefore exhibit significant host toxicity. Amphotericin B is another drug used to treat antimony-resistant leishmaniasis, but it too has severe side-effects. (Borges et al. 2017) A non-antimony-based compound, miltefosine, has been successfully employed as an oral treatment for leishmaniasis. (Kuhlen cord et al. 1992) However, resistance to miltefosine has been reported, both in the lab and in clinical leishmaniasis cases. (Srivastava et al. 2017) Efforts are also underway to develop vaccines against *Leishmania* parasites, but thus far no effective vaccines are available. (Gillespie et al. 2016; Reed et al. 2016; Srivastava et al. 2016) In order to better treat leishmaniasis, current research in the Beverley laboratory aims to understand the basic biology of *Leishmania* and leverage that knowledge to identify potential drug targets.

**1.2– Leishmaniavirus: Infection-in-an-Infection**

Within the last 40 years, it has been discovered that several strains of *Leishmania* are infected with dsRNA viruses. The first of these was discovered in *Leishmania braziliensis* in the 1980s, based on the presence of a large RNA species associated with virus-like particles. (Tarr et al. 1988) Shortly thereafter, other virus strains were discovered in *L. braziliensis*, *L. guyanensis*, and *L. major*. (Widmer et al. 1989; Scheffter et al. 1995; Cadd et al. 1993) These viruses were all grouped into a new genus, *Leishmaniavirus*, within the family Totiviridae. Totiviruses are defined by their single-segmented dsRNA genomes (Figure 1), which encode a capsid protein and an RNA-dependent RNA polymerase (RDRP). To aid in subsequent *Leishmaniavirus*
screening efforts, we have developed a suite of additional methods for identifying *Leishmaniavirus* in parasites. These approaches are described in detail in Chapter 2. (Zangger et al. 2013) Most notably, we optimized dot-blot and slot-blot assays utilizing antibodies raised against dsRNA, which allowed us to detect the dsRNA viral genome in a sequence-independent manner. This technique may be suitable for use in the field to analyze clinical isolates of *Leishmania*. Subsequent large-scale screening in the Beverley laboratory has shown that approximately 30% of clinical isolates from South America contain *Leishmaniavirus* (unpublished).

Members of the genus *Leishmaniavirus* are divided into two species, *Leishmania* RNA Virus 1 and 2 (LRV1 and LRV2), on the basis of sequence divergence and genome arrangement (Figure 2). LRV1 includes multiple genetically similar virus strains that have been identified in various isolates of *L. guyanensis* and *L. braziliensis*. LRV2 has only been found in a few isolates of *L. major* and *L. aethiopica*. (Zangger et al. 2014; Scheffter et al. 1995). The most notable difference between LRV1 and LRV2 is the relative reading frames of the capsid and RDRP genes’ open reading frames (ORFs). In LRV1 the RDRP ORF is shifted +1 relative to the capsid ORF, a feature which has been previously observed for a few other members of the Totiviridae family. (Stuart et al. 1992; Scheffter et al. 1994; Dinman et al. 1991) The LRV2 strain isolated from *L. major* has both ORFs in the same reading frame, separated by a stop codon. (Scheffter et al. 1995) The other LRV2 strain, from *L. aethiopica*, has a -1 frameshift. (Zangger et al. 2014) The significance of these various ORF arrangements is unclear. *Leishmaniavirus* is only transmitted vertically from mother to daughter cells during cell division. This results in specific virus-host pairings where each infected *Leishmania* strain has a corresponding *Leishmaniavirus* strain. (Widmer and Dooley 1995) The best-studied of these virus strains, LRV1-Lgy-M4147,
infects *L. guyanensis* MHOM/BR/75/M4147. (Scheffler et al. 1994) Most of the experiments described in this dissertation make use of this particular model LRV1 strain and its corresponding *Leishmania* strain.

More recently, screening of *Leishmania* isolates for unknown large dsRNA species has led to the discovery of another, unrelated virus genus, *Leishbunyavirus* (LBV). LBV is a tri-segmented negative sense single-stranded RNA virus in the bunyavirus family. We have found that LBV is both more common than LRV and more effective at increasing pathology and parasite burden in mouse models of leishmaniasis. Thus, LBV may ultimately prove to be more clinically relevant than LRV1. In Appendix A I describe preliminary work on a drug screen allowing LBV inhibitors to be identified at the same time as *Leishmania* and LRV1 inhibitors.

1.3 – *Leishmaniavirus* Life Cycle Depends on its RNA Polymerase

With the exceptions of the genus *Giardiavirus* (Miller et al. 1988) and an unclassified Totivirus infecting Atlantic salmon, (Lovoll et al. 2010) Totiviruses are non-infectious and their entire life-cycle is intracellular. Viruses are only passed between cells during mating and cell division. (Wickner et al. 2013) Recent work by our collaborator, David Sacks, has confirmed this for LRV2, which can be passed between *L. major* strains during mating in the sand fly midgut. (Owens, Lye, Inbar, Akopyants, Sacks, Beverley; unpublished data) Congruency of the virus and parasite phylogenetic trees suggests that the members of *Leishmaniavirus* have been transmitted vertically for more than 50 million years, prior to the divergence of the Old and New World *Leishmania* species. (Widmer and Dooley 1995) The persistence of LRV infection is such that, prior to this work, LRV+ parasites in culture had only been observed to lose their infection on one occasion, and this has not been replicated despite direct efforts to eliminate the virus. (Ro et al. 1997) Furthermore, attempts to directly electroporate purified virions into different
Leishmania strains have only yielded very transient infections where the virus is quickly lost. This suggests strong host-virus specificity. (Armstrong et al. 1993) Interestingly, the number of viruses per parasite cell appears to be tightly regulated. (Weeks et al. 1992) The mechanism behind this regulation has not yet been conclusively determined. In Chapter 3 of this dissertation, we quantified the normal virus titer to be \(15 \pm 0.9\) virions per cell.

As a member of the family Totiviridae, the Leishmaniavirus lifecycle (Figure 3) is similar to that of the type species and best-characterized member of this family, the L-A virus from Saccharomyces cerevisiae. (Wickner et al. 2013) Electron microscopy of other Totiviruses suggests that the over-all virion structure is largely conserved throughout the family. (Parent et al. 2013; Dunn et al. 2013) Mature virions contain the dsRNA viral genome plus 1-3 RDRPs, possibly as capsid-RDRP fusion proteins. Capsid-RDRP fusions have been observed in other members of the family Totiviridae. The overlap of the LRV capsid and RDRP ORFs, combined with the absence of a Kozak sequence for the RDRP gene and a sequence in the overlap region that is known to cause ribosomal frameshifting, support such a mechanism for LRV, although this has never directly demonstrated. (Lee et al. 1996) However, a related translation stop-start mechanism is known to occur in other Totivirus species. (Dinman et al. 1991; Parent et al. 2013)

None of the high-resolution Totivirus virion structures solved to date include the RDRP structure, likely because the RDRP is present at only a few copies per virion. As a result, its average occupancy of any one vertex would be too low for it to appear in complete virion structures. (Parent et al. 2013; Naitow et al. 2002; Dunn et al. 2013; Janssen et al. 2015) The RDRPs transcribe the dsRNA genome to create new positive-sense viral genomic transcripts, extruding them out of the virion.
These transcripts are then translated by the *Leishmania* ribosome to produce capsid and RDRP for constructing new virions. L-A virus “snatches” caps from cellular mRNAs to install on a fraction of its own transcripts, allowing for standard cap-dependent translation. (Fujimura and Esteban 2011) Interestingly, early work on LRV1 found that most viral genomes terminated in a phosphate group on their 5' ends, suggesting that LRV1 may not snatch caps, but capped viral genomes and cap-snatching activity have not been directly assayed. (Widmer et al. 1989) However, LRV does contain an internal ribosome entry site (IRES) in its 5' untranslated region (UTR) which may allow it to be translated in the absence of a 5' 7mGppp cap. (Stuart et al. 1992; Maga et al. 1995)

Once translated, the capsid monomers and capsid-RDRP fusion complex self-assemble to form a new virion. Evidence from the L-A virus literature suggests that newly synthesized capsid-RDRP fusions bind free positive-sense viral transcripts and package them into new virions. In L-A virus, this is mediated by a conserved RNA stem-loop structure near the 3' end of the positive strand of its genome, but no experiments have probed the corresponding sequence in LRV. (Esteban et al. 1988) Once packaged into a virion, the RDRP recognizes a replication signal near the 3' end of the packaged positive-sense viral genomic RNA and replicates it to produce the mature dsRNA genome. (Weeks et al. 1992; Esteban et al. 1989) Interestingly, the LRV capsid protein contains an endonuclease activity which cleaves the viral genome at a specific site, likely destroying it. This is thought to play a role in controlling the viral titer, although the lack of a reverse genetic system for LRV1 has prevented direct proof. (Ro and Patterson 2000; Ro et al. 2004; Ro and Patterson 2003; MacBeth et al. 1997; Macbeth and Patterson 1998; MacBeth and Patterson 1995)
1.4--LRV has Clinical Relevance

When they were first discovered, the biological significance of LRV1 and LRV2 was unclear. One isogenic pair of infected and uninfected parasites was fortuitously generated, but the parasites' phenotypes in culture were identical, with no apparent effect on growth or viability. (Ro et al. 1997) However, in 2011 our lab, in collaboration with Dr. Fasel's group, demonstrated that the presence of LRV1 in parasite strains is correlated with the severity of disease caused by those strains. In a mouse model of leishmaniasis, LRV-positive parasites induce a TLR3-dependent type I interferon response more typical of viral infection than parasite infection. The mice show a significantly greater parasite burden and more frequent metastasis from the infected footpad to the tail. TLR3 is an innate immune sensor of dsRNA located in the endosomal compartment. (Jensen and Thomsen 2012) Since *Leishmania* parasites take up residence in host macrophage endosomes, we theorize that macrophages kill parasites early in infection and liberate the LRV1 dsRNA genome. This would be detected by TLR3 and initiate the innate immune responses to viral infection. This response includes up-regulation of pro-inflammatory cytokines, such as TNF-α and IL-6, and the type-1 interferon IFN-β. These signaling molecules would then induce inflammation and exacerbate disease severity. (Ronet et al. 2011; Hartley et al. 2012; Ives et al. 2011) Subsequent work has shown that this increased disease phenotype can be replicated by co-infecting mice with LRV1-negative *Leishmania* and a murine virus known to induce type I interferon. It can also be replicated by stimulating type I interferon directly in *Leishmania*-infected mice. (Rossi et al. 2017) Intriguingly, LRV1-dependent immune effects can be blocked by immunizing mice with the LRV1 capsid protein. (Castiglioni et al. 2017) This suggests that it may be possible to block the deleterious effects of LRV1 in human leishmaniasis by inhibiting LRV1 or immunizing against it.
Although this mechanism of immune modulation by a protozoan virus was novel at the time, it has since been observed in *Trichomonas* infections. Some strains of *Trichomonas* are infected by Totiviruses from the closely-related *Trichomonasvirus* genus.(Goodman et al. 2011a; Janssen et al. 2015; Bessarab et al. 2000; Parent et al. 2013; Liu et al. 1998; Goodman et al. 2011b) As with *Leishmania*, these strains induce a more severe disease phenotype.(El-Gayar et al. 2016; Wendel et al. 2002; Fraga et al. 2007; Fichorova et al. 2012)

Following the discovery of LRV1-dependent disease exacerbation in mice, multiple groups searched for a corresponding effect in human leishmaniasis. In collaboration with several other groups, we showed that the presence of LRV1 in *L. guyanensis* correlates with the severity of CL.(Hartley et al. 2016) Related work has shown that the presence of LRV1 increases treatment failure and relapse rates in *L. guyanensis* and *L. braziliensis* cases.(Adaui et al. 2015; Ito et al. 2015; Cantanhede et al. 2015; Bourreau et al. 2015) Initially, we suspected that LRV1 would be strongly linked to MCL, given the vigorous inflammatory response characteristic of that form of leishmaniasis. However, although some studies found that LRV1-positive strains generate higher rates of MCL (Hartley et al. 2014; Cantanhede et al. 2015; Bourreau et al. 2015), other studies failed to identify a correlation.(Adaui et al. 2015; Periera et al. 2013) In mouse models of leishmaniasis, LRV1 has a more substantial effect on parasite number than on host immune response. Since MCL is believed to depend more on host immune responses than on parasite load, one would expect that the correlation between LRV1 and MCL would be weaker than the one between LRV1 and CL severity.(Hartley et al. 2014; Banuls et al. 2011; Mears et al. 2015; Goto and Lindoso 2010; Guerra et al. 2011)
1.5 – Purine Metabolism in *Leishmania*: an Enticing Drug Target

Given the importance of LRV1 as a determinant of leishmaniasis severity, we began developing methods to eliminate it from parasites. Initially, we utilized the RNAi pathway present in *Leishmania (Viannia)* species to knock down and eventually eliminate LRV1. This was accomplished by introducing a strongly transcribed stem-loop construct containing LRV1 sequences into LRV1-positive parasites. The large amount of dsRNA produced would be processed by the RNAi pathway and used to target and degrade viral RNAs. This method was successful, but also cumbersome and unsuitable for use in the clinic. (Brettmann et al. 2016) We then turned to small molecule anti-viral drugs. Since the LRV1 RDRP is the main driver of viral replication, we focused on nucleoside analogs, which are the most common class of RNA polymerase inhibitors.

Another reason to investigate nucleoside analog LRV1 inhibitors is that *Leishmania* parasites are purine auxotrophs, and thus avidly scavenge purine nucleosides and nucleobases from the extracellular milieu. These salvage pathways make obvious targets for small molecule *Leishmania* inhibitors. This approach has been partially successful – allopurinol, an analog of hypoxanthine, is selectively phosphorylated by the *Leishmania* purine salvage pathway and incorporated into parasite RNAs, where it is thought to exert its toxic effects. (Marr 1983) Allopurinol can be used to treat leishmaniasis in humans. (Martinez and Marr 1992; Marr and Berens 1977) However, associated side-effects have limited the practical use of allopurinol to treatment of leishmaniasis in dogs, an important reservoir species in South America. (Reguera et al. 2016) More recent efforts have revealed that a class of nucleoside analogs called immucillins can inhibit *Leishmania* parasites and treat leishmaniasis in a mouse model of infection. (Freitas et al. 2015a) *In vitro* experiments show that these compounds act on a non-specific nucleoside
hydrolase that is key to the nucleoside salvage pathways, but other “off-target” effects may account for their potency against parasites in vivo. (Shi et al. 1999; Freitas et al. 2015b) These compounds have not yet been employed against human leishmaniasis.

The *Leishmania* purine salvage pathway is a tightly-interconnected network of enzymes capable of converting every standard nucleoside into any other (Figure 4). (Boitz and Ullman 2013; Carter et al. 2008) Extracellular nucleotides are scavenged by multiple extracellular phosphatases and nucleotidases that hydrolyze them to the nucleoside level prior to uptake. (Freitas-Mesquita and Meyer-Fernandes 2014) Parasites express multiple transporters with varying specificities that import the nucleosides. (Sanchez et al. 2004; Aronow et al. 1987) Nucleosides entering the parasite are initially degraded to nucleobases before entering the salvage pathway. This is accomplished by a number of nucleoside hydrolases, including a non-specific enzyme that acts on all naturally occurring purine and pyrimidine nucleosides. (Shi et al. 1999; Cui et al. 2001) The nucleobases are then converted to nucleoside monophosphates via combination with phosphoribosyl pyrophosphate. (Boitz and Ullman 2006) Salvage of adenosine, unlike the other nucleosides, can also proceed directly from adenosine to adenosine monophosphate via an adenosine kinase enzyme. (Datta et al. 1987; Bhaumik and Datta 1988; Iovannisci and Ullman 1984) However, most adenosine is instead hydrolyzed to adenine and then converted to hypoxanthine by adenine aminohydrolase. (Boitz et al. 2012) The core of the purine salvage network is a set of enzymes that interconvert nucleoside monophosphates to balance the adenylate and guanylate nucleotide pools. (Boitz et al. 2016) These are localized in the glycosome, an organelle unique to *Leishmania* and its relatives. (Shih et al. 1998; Colasante et al. 2013; Oppendoes and Szikora 2006)
1.6– Aims and Scope

Given that the presence of LRV1 in human leishmaniasis cases significantly increases disease severity and negative treatment outcomes, an inhibitor of LRV1 replication could be useful in the clinic. The primary aim of this work is to identify such inhibitors, determine their modes of action, and suggest ways to improve their potency.

Before setting out to identify LRV1 inhibitors, I developed a method for efficiently measuring the amount of dsRNA in Leishmania cells (Chapter 2). Since LRV accounts for most dsRNA in LRV+ parasites, this allowed me to estimate the amount of LRV1 per parasite. (Zangger et al. 2013) Using this technique and a previously-developed flow cytometry assay of LRV1 capsid levels, Dr. F. Matthew Kuhlmann and I screened a panel of purine analogs for activity against LRV1. In Chapter 3, I describe the screen and the two effective inhibitors we identified, 2’-C-methyl-adenosine (2CMA) and its close relative 7-deaza-2CMA (7d2CMA). Using these drugs, we were able to cure Leishmania cultures of LRV1.

Next, I investigated the mechanism of action of 2CMA. Because 2CMA and 7d2CMA are adenosine analogs known to inhibit the Hepatitis C virus RDRP, I hypothesized that the drug triphosphosphate (2CMA-TP) would inhibit the LRV1 RDRP. (Carroll et al. 2003; Olsen et al. 2004) In Chapter 4, I describe experiments which demonstrate that 2CMA-TP is the active form of 2CMA and that it inhibits the LRV1 RDRP. Unexpectedly, the IC50 of 2CMA-TP in vitro was much higher than the EC50 of 2CMA in vivo. I show that this occurs because the parasites accumulate high concentrations of 2CMA-TP. Appendix A describes preliminary genetic and pharmacological manipulations I performed with the goal of improving the nucleoside analog sensitivity of L. guyanensis enough to enable testing their efficacy in our murine leishmaniasis
model. Appendix B contains pharmacokinetic calculations for 7d2CMA, plus several experimental options for testing the effects of treating LRV1 in mice with 7d2CMA.

The work summarized herein contributes to our ability to study the effects of LRV1 on *Leishmania* and on host immune responses to *Leishmania* by allowing us to quickly and easily eliminate LRV1 from parasites. The unexpected role of purine salvage in the effectiveness of 2CMA provides insight into the importance of that pathway in drug design for *Leishmania*. Forthcoming experiments in our mouse model of leishmaniasis should determine whether chemotherapy of LRV1 would improve disease progression. Ultimately, this knowledge may enable the development of anti-LRV1 therapies capable of improving treatment outcomes in human leishmaniasis, whether by vaccination or inhibition with nucleoside analogs.
1.7–References


associated with Leishmania RNA virus 1 infection in Brazil. Mem Inst Oswaldo Cruz 108 (3):1-3


1.8 – Figures

Figure 1-1: Genome of LRV1 from L. guyanensis M4147.

The 5' untranslated region (UTR) can function as an internal ribosome entry site (IRES).

In this strain, the RDRP open reading frame is shifted +1 relative to the capsid.
Figure 1-2: Genomic arrangement and phylogeny of LRV strains.

The tree was constructed with ClustalX in UPGMA mode, using L-A virus as an outgroup (not shown) (Larkin et al. 2007). The bars illustrate the coding frame of the RDRP open reading frame (black) relative to the capsid (white).
Figure 1-3: LRV life cycle.

The RDRP (black trapezoid) transcribes the mature dsRNA genome to produce the positive-sense transcript (red), which is extruded from the viral capsid. The host ribosome translates the transcript, producing new capsid monomers (circles) and capsid-RDRP fusion proteins. Note that for LRV the fusion protein is hypothetical and has not been observed in vivo. These assemble to form a new virion, packaging a viral transcript. The RDRP replicates the viral transcript, forming the negative-sense strand (blue) and resulting in a new mature virion.
Diagram is based on data from the KEGG database and visualized in VANTED. (Kanehisa et al. 2017; Rohn et al. 2012) Compounds are unboxed text and enzymes are boxed. Nucleosides (bottom row) are taken up into *Leishmania* cells where they are hydrolyzed by a nucleoside hydrolase (NH) to nucleobases. Nucleobases are converted to nucleoside monophosphates by various phosphoribosyl transferase (PRT) enzymes. From here a host of enzymes facilitates interconversion between the adenylate and guanylate nucleotide pools. Note that adenosine can be directly phosphorylated by adenosine kinase (AK).
Chapter 2: Detection of *Leishmania* RNA Virus in *Leishmania* Parasites
Preface


Specifically, JIR designed and optimized the dsRNA slot-blot assay, analyzed the data, and created the associated figure with extensive technical and conceptual assistance from SMB and FMK.

This chapter was previously published:

2.1 – Abstract

2.1.1 – Background

Patients suffering from cutaneous leishmaniasis (CL) caused by New World *Leishmania* (*Viannia*) species are at high risk of developing mucosal (ML) or disseminated cutaneous leishmaniasis (DCL). After the formation of a primary skin lesion at the site of the bite by a *Leishmania*-infected sand fly, the infection can disseminate to form secondary lesions. This metastatic phenotype causes significant morbidity and is often associated with a hyper-inflammatory immune response leading to the destruction of nasopharyngeal tissues in ML, and appearance of nodules or numerous ulcerated skin lesions in DCL. Recently, we connected this aggressive phenotype to the presence of *Leishmania* RNA virus (LRV) in strains of *L. guyanensis*, showing that LRV is responsible for elevated parasitaemia, destructive hyper-inflammation and an overall exacerbation of the disease. Further studies of this relationship and the distribution of LRVs in other *Leishmania* strains and species would benefit from improved methods of viral detection and quantitation, especially ones not dependent on prior knowledge of the viral sequence as LRVs show significant evolutionary divergence.

2.1.2 – Methodology/Principal Findings

This study reports various techniques, among which, the use of an anti-dsRNA monoclonal antibody (J2) stands out for its specific and quantitative recognition of dsRNA in a sequence-independent fashion. Applications of J2 include immunofluorescence, ELISA and dot blot: techniques complementing an arsenal of other detection tools, such as nucleic acid purification and quantitative real-time-PCR. We evaluate each method as well as demonstrate a successful LRV detection by the J2 antibody in several parasite strains, a freshly isolated patient sample and lesion biopsies of infected mice.
2.1.3 – Conclusions/Significance

We propose that refinements of these methods could be transferred to the field for use as a diagnostic tool in detecting the presence of LRV, and potentially assessing the LRV-related risk of complications in cutaneous leishmaniasis.

2.1 – Introduction

Leishmaniasis is one of the most important human protozoan parasitic diseases worldwide, with a prevalence of 12 million infections and a further 350 million people living at risk across 98 countries [1], [2]. It mainly presents in two major clinical forms: 1) cutaneous leishmaniasis (CL) in which lesions are generally localized and self-healing or 2) visceral leishmaniasis (VL) known to fatally disseminate to viscera. CL can be caused by various species, either from the Leishmania (Leishmania) subgenus (e.g. L. major, L. mexicana and L. amazonensis) or members of the L. (Viannia) subgenus (e.g. L. braziliensis, L. panamensis and L. guyanensis), while VL is mostly attributed to L. donovani, L. infantum and L. chagasi. Beyond the intrinsic parasite factors that seem to determine disease phenotype, extrinsic factors within the host are also known to alter the symptomatic spectrum of leishmaniasis [3].

In South America, CL patients mainly infected by L. braziliensis, L. panamensis and L. guyanensis are at risk for developing mucosal (ML) or disseminated cutaneous leishmaniasis (DCL) [3], [4], [5], [6], which are complications of CL involving dissemination of parasites from primary lesions to secondary sites, with or without mucosal involvement, and causing lesions that are often associated with a highly destructive inflammatory response [7], [8], [9], [10]. Mucosal disease is notorious for its poor response to commonly used treatments, such as antimony, and is often complicated by secondary bacterial or fungal infections. Very little is known about the pathogenesis of metastatic and mucosal leishmaniasis; especially the source of
the uncontrolled inflammatory response observed in some patients. Two factors that have been associated with mucosal and disseminated disease include host genetic polymorphisms (e.g. in TNF, IL-6 and HLA genes) and HIV co-infection [11], [12], [13].

Recently, we suggested that the presence of a parasite dsRNA virus could contribute to the severity of the disease in strains of L. guyanensis [14], [15], [16]. This Leishmania dsRNA virus (LRV) has been found in various L. (Viannia) species as well as in one L. major strain [17]. Notably, in murine models of L. guyanensis infection, the LRV dsRNA genome is innately recognized by host Toll-like-receptor (TLR3), exacerbating the disease in a dose-dependent manner [14], [15].

Leishmania has a digenetic life cycle, with a motile extracellular promastigote form in the midgut of a female sand fly, and a non-motile intracellular amastigote form in the mammalian host macrophage. Our model proposes that the innate recognition of LRV takes place in the first few hours of infection. Here, some fraction of parasites die, releasing viral dsRNA that then binds to Toll-like receptor 3 (TLR3) trigging the subsequent IFN-type I driven inflammatory cascade that worsens disease [14], [18]. A high LRV burden in infecting parasites could therefore be a major determinant of disease severity and pathology.

LRV is a member of the Totiviridae family that regroups viruses found in several kingdoms of life [16], including protozoan parasites such as Giardia, Trichomonas vaginalis, fungi such as Helminthosporium sp. and S. cerevisiae as well as mosquitoes [19] and salmon [20]. They are small and simple virions (30–50 nm), containing a dsRNA genome that encodes its single capsid protein and an RNA-dependant RNA polymerase (RdRp), necessary and sufficient for both viral genomic dsRNA replication and viral ssRNA transcription. Viral transcripts are translated in the host cell cytoplasm into a capsid protein and, in most Totiviridae,
into a fusion capsid-RdRp polypeptide (82 kDa and 176 kDa respectively). According to detailed studies in yeast, a single virion is composed of more than a hundred capsid protein molecules and one to two capsid-RdRp subunits surrounding the single genomic dsRNA molecule [21].

LRVs were identified and characterized several years ago in *L. (Viannia) braziliensis* and *guyanensis* [22], [23], [24] as well as in a single isolate of *L. major* [17]. Although their genomic organization is identical, high diversity in nucleotide sequence (less than 40% homology according to [17]) between LRVs of *L. (Viannia)* and *L. major* has categorized *Leishmania* viruses into the groups LRV1 and LRV2 respectively [17].

An important finding from our prior work is that only parasites with high levels of LRV exacerbated disease severity [14], [15], and previous studies have shown that considerable diversity in sequence is found amongst LRVs [17]. Studies looking into the role of LRV would thus be greatly aided by the availability of diverse methods for LRV detection and quantification, especially simple, rapid and reliable techniques, suitable for screening a large number of parasite strains in the field. To this end, we used parasite strains bearing different levels of LRV as standards [14]. Reliable detection and quantification was achieved by dsRNA extraction, quantitative real-time PCR (qRT-PCR) as well as with the immuno-detection of LRV genome in lysed, fixed or live parasite samples (ELISA, dot blot and fluorescence microscopy). Although qRT-PCR can be used efficiently and is a powerful method for detailed molecular studies on reference strains, it could have limited application for LRV screening on uncharacterized parasites from the field due to possible nucleotide and amino acid polymorphisms of LRVs. This problem was addressed by focusing on detection of dsRNA through the use of an anti-dsRNA monoclonal antibody (J2), which specifically recognizes dsRNA independent of its underlying nucleotide sequence. We applied this approach on several catalogued human isolates, on a
fresh *L. braziliensis* sample obtained from a patient as well as on murine lesions biopsies, showing the relative ease of use of these methods for field application. We propose the technique as having great diagnostic potential for predicting the LRV-related risk of leishmanial dissemination.

### 2.2 Methods

#### 2.2.1 Ethical statement

This study was approved by the Ethics Committee of Canton of Vaud, Lausanne (Switzerland) for the analysis of *Leishmania* parasites isolated from patients. The two *L. braziliensis* parasites strains (MHOM/BO/2011/2169 and MHOM/BO/2011/2192) were isolated from a *L. braziliensis*-infected patient, who signed an informed consent accepting the use of materials for publication. Other *Leishmania* parasite strains used in this study are common lines isolated several years ago and described in several reports.

#### 2.2.2 Parasite strains and cultures

Different *L. guyanensis* reference strains of known LRV content [14] were used: i) two clones derived from the M4147 population (MHOM/BR/75/M4147) infected or not by LRV designated here as *Lg* M4147 LRV$_{\text{high}}$ (M4147/SSU:IRSAT-LUC(b)) and *Lg* M4147 LRV$_{\text{neg}}$(M4147/pX63HXG/SSU:IRSAT-LUC(b)) respectively [25], ii) human isolates of *L. guyanensis* *Lg*1398 (MHOM/BR/89/IM3597) and *Lg* 1881 (MHOM/BR/92/IM3862) and iii) *L. guyanensis* M5313 parasites (WHI/BR/78/M5313) and their derived non-metastatic (*Lg* 03 and *Lg* 17) or metastatic (*Lg* 13 and *Lg* 21) clones [14], [26]. Five human isolates of *L. braziliensis*, previously shown to be LRV-infected [24], were also analyzed: MHOM/CO/88/1407C (*Lb* 1407C), MHOM/CO/88/1407M (*Lb* 1407M), MHOM/CO/88/1403 (*Lb* 1403), MHOM/CO/86/1174 (*Lb*1174) and MHOM/CO/84/1064 (*Lb* 1064). Two strains of *L.
braziliensis parasites were freshly isolated from an infected patient who contracted leishmaniasis: MHOM/BO/2011/2169 (from primary cutaneous lesion) and MHOM/BO/2011/2192 (from secondary/metastatic lesion), referred to in the text as Lb 2169 and Lb 2192.

Parasites were cultivated as promastigotes at 26°C in freshly prepared Schneider's insect medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (PAA), 10 mM Hepes (Amimed), 50 U/ml penicillin/streptomycin (Amimed), 0.6 mg/L biopterin (Sigma) and 5 mg/L hemin (Sigma).

2.2.3 – Viral dsRNA extraction from total nucleic acids

Stationary phase Leishmania promastigotes were lysed for 20 min at RT with 0.4% sarkosyl and protease inhibitors (Roche) diluted in 1×PBS (10^8 parasites in 100 µl). The lysates were then incubated at 37°C, first for 30 min with 400 µg/ml of recombinant proteinase K (Roche), then for a further 2 h with 10 µg/ml RNase (DNase-free from Roche). Nucleic acids, containing genomic parasitic DNA and LRV dsRNA, were extracted from these lysates by phenol-chloroform (at least twice), precipitated with 0.3 M sodium-acetate in 70% ethanol, then washed and resuspended in water (approx. 20 µl for 10^8 parasites). DNA was quantified by spectrophotometry (Nanodrop). Pure viral dsRNA was obtained after RQ-DNase digestion according to manufacturer's instruction (Promega). Nucleic acids were analysed on 0.6% to 1.2% agarose gels containing SYBR-safe for nucleic acid staining (Invitrogen).

2.2.4 – Quantitative real-time PCR (qRT-PCR)

RNA was extracted from stationary phase promastigotes (approx. 3×10^7) using Trizol (Invitrogen) according to manufacturer's instruction (1 ml Trizol for a 1 ml promastigote culture). After extraction, precipitation and washing, RNA was resuspended in water
(3×10^7 parasites in 10 µl) and quantified by spectrophotometry. 0.5–1 µg of RNA was then used
for cDNA synthesis with SuperScript II Reverse Transcriptase (Invitrogen), which was finally
purified with a QIAquick PCR purification kit (Qiagen). qRT-PCR was undertaken in a reaction
solution of 0.5 µM primer diluted in SYBR Green Master mix (LightCycler 480 system, Roche).
The reaction consisted of an initial denaturation at 95°C for 5 min followed by 40 cycles of
amplification: 10 s at 95°C, 10 s at 60°C, 10 s at 72°C and a fluorescence detection step at 78°C
to quantify the amplified DNA after each cycle. The following DNA oligonucleotides
(Microsynth, Switzerland) were used: SetA: 5′-CTG ACT GGA CGG GGG GTA AT-3′ and 5′-
CAA AAC ACT CCC TTA CGC-3′/SetB: 5′-GTC TGT TTC GTA CCC GCC G-3′ and 5′-AAG
CTC AGG ATG TGC ATG TTC CA-3′/kmp11 specific primers: 5′-GCC TGG ATG AGG AGT
TCA ACA-3′ and 5′-GTG CTC CTT CAT CTC GGG-3′. SetA and SetB were based on LRV1-4
genome sequence (GenBank accession number: NC003601) and L. major kmp11 gene as
described previously [14]. LRV transcript levels were quantified in triplicate relative to
the Leishmania kmp11 housekeeping gene. Analysis and acquisition of data were performed with
the LightCycler software 1.5 (Roche) using the 2^-ΔΔCT method.

2.2.5 – Anti-capsid antibody production and immunoblotting

The LRV capsid open reading frame was amplified from a cDNA preparation
of Lg M5313 and cloned in a pET-28a E. coli expression vector (Merck). Its sequence was found
to be highly similar to the capsid sequence of Lg M4147 LRV1-4 (more than 98% identical
residues, Genbank accession number: JX313126). Recombinant capsid was purified, using a
HIS-tag, then used for rabbit immunization (Covalab, polyclonal antibody identification code:
g018d53). Proteins from total parasite extracts were quantified by BCA, 40 µg was loaded and
separated on a 10% polyacrylamide denaturing gel, transferred to a nitrocellulose membrane and
visualised by Ponceau Red staining. After a 1 h blocking step in 5% powdered milk diluted in TBS+0.05% Tween20, the membrane was incubated overnight at 4°C with the g018d53 anti-capsid polyclonal antibody (1:5000 in 1% milk TBS-Tween20). Following 4 washes of 15 min at RT, the membrane was incubated for 1 h with an anti-rabbit IgG antibody coupled to peroxidase (Promega) (1:2500 in 1% milk TBS-Tween20), washed again 4× and finally revealed by ECL chemiluminescence (Amersham).

2.2.6 – Peptide arrays on cellulose membranes and epitope mapping

For antibody epitope screening, seventy-four 20-mer overlapping peptides (with an overlap of 10 residues) that cover the whole sequence of Lg M4147 LRV1-4 capsid (Genbank accession number: NC003601) were synthesized and attached to cellulose membranes by the Protein and Peptide Chemistry Facility (University of Lausanne).

The peptides were synthesized by using Intavis MultiPep synthesizer (Intavis Bioanalytical Instruments AG, Cologne, Germany). The cellulose membrane used was an Amino-PEG500-UC540 sheet (acid-hardened with improved stability). The membrane peptide linker was stable in wide range of aqueous pH (pH 0–pH 14) at ambient temperature for 12 hours. The PEG spacer consisted of 8–10 ethylene glycol units and had free terminal amino groups to start the peptide synthesis. The Amino-PEG500 spacer was loaded at 400 nmol/cm² with a typical spot diameter of 4 mm and therefore an average of 50 nmol peptide/spot. The peptides were synthesized by stepwise solid phase synthesis. Amino acids that had N-terminal/side-chain protecting groups were spotted (if required) by robot. The amino acid solutions were activated using diisopropylcarbodiimide/hydroxybenztriazole chemistry. For each cycle, solutions of the 20 common amino acids were dispensed along with solutions of modified amino acids as required (e.g. phosphorylated amino acids, acetylated amino acids,
methylated amino acids). Following addition of the first amino acids, the membranes were treated to prepare the spots for the next in sequence. This was done by removing the N-terminal protective group (Fmoc) by piperidine. This cycle was repeated until the peptides reached the required length. Arrays were then treated with trifluoroacetic acid to reveal the native side chains. Arrays were stored at −20°C prior to use.

Similarly to the classic nitrocellulose membranes as described above, these peptide-spotted membranes were incubated with the g018d53 anti-capsid polyclonal antibody (1∶5000) to allow the determination of the epitopes for which it was specific.

2.2.7 – LRV sequencing

*Lg* 1398 LRV genome was partially sequenced as follows: first, viral dsRNA was obtained from approximately 10⁹ stationary phase promastigotes after total nucleic acids extraction and RQ-DNase digestion of genomic DNA (see “Viral dsRNA extraction from total nucleic acids” section) and purification of the 5.3 kb band after 0.8% agarose gel electrophoresis using Wizard SV gel and PCR clean-up system (Promega). Viral cDNA was then synthesized as described above (“Quantitative real-time PCR” section) and 10–50 ng was used for PCR amplification with 0.4 µl of GoTaq DNA polymerase (Promega) in its buffer supplemented with Q solution (Qiagen), 0.4 mM dNTPs (Promega) and 0.3 µM of each oligonucleotides (Microsynth, Switzerland). The PCR reactions consisted of 35 cycles: 1 min at 94°C, 1 min at 55°C and 2 min at 72°C. Two PCR fragments were generated and sequenced (by Fasteris, Switzerland) with the following oligonucleotides: i) 5’-GGA TCC GAA ACG TAA GCA AGT TTC TTG-3’ and 5’-CCA ATA CCA TGG CGC CAT CAC ATT CAT-3’ (based on LRV1-1 and 1-4 sequences) and ii) 5’-GAG AAA TAG CGA TAT CGC AGC CCA A-3’ (based on *Lg* 1398 LRV sequence obtained from previous reaction) and 5’-CAC AGC CAA CGT GAC GAC CAG
AAA TCA C-3’ (LRV1-4). These two products allowed us to obtain 3.3 kb of Lg 1398 LRV genome sequence including the complete open reading frame of the viral capsid.

2.2.8 – Immunofluorescence microscopy (IFM)

Two different protocols were used. In protocol A, stationary phase promastigotes were fixed with 4% formaldehyde in PBS for 20–30 min (or overnight in 1% at 4°C), washed and resuspended at 2×10⁷ parasites/ml then attached to poly-lysine (Sigma) coated slides (Thermo Scientific) for 30 min at RT. After a 10 min permeabilization step in PBS+0.1% TritonX-100 (PBS-TX), cells were blocked for 45–60 min in 2% bovine serum albumin (BSA, Acros Organics) in PBS-TX, and incubated overnight at 4°C with the rabbit g018d53 anti-capsid polyclonal antibody (1:5000) or the mouse anti-dsRNA J2 antibody (1:800, English & Scientific Consulting) in 1% BSA in PBS-TX. Cells were then washed 4× in PBS, incubated for 1 h with a goat anti-rabbit IgG coupled to Alexa Fluor 594 (1:2000, Invitrogen) or a goat anti-mouse antibody coupled to Alexa Fluor 488 (1:600, Invitrogen) in 1% BSA in PBS-TX. These were washed twice, incubated 10–30 min with 0.5 µg/ml 4’6-Diamidino-2-phenylindole (DAPI, Invitrogen), washed again and finally mounted with Vectashield diluted 100× in DABCO mounting solution (90% glycerol, 10% PBS and 2.5% DABCO from Sigma) or using Permafluor (ThermoScientific). Fluorescence visualization was performed with an Upright Axio Microscope at the Cellular Imaging Facility (CIF Epalinges, University of Lausanne).

In protocol B, 10⁶ parasites were fixed with 2% paraformaldehyde in PBS for 2 min. Cells were washed once in PBS and adhered to glass coverslips (Fisherbrand) by centrifugation (500 g for 2 min). Cells were permeabilized in blocking buffer (5% normal goat sera, 0.1% Triton-X100, 1× PBS) for 30 min at room temperature then incubated with mouse anti-dsRNA J2 antibody (1:1000) for one hour. Cells were then washed 3× in PBS and incubated with goat
anti-mouse IgG AlexaFluor 488 (1:1000, Invitrogen) for one hour. After washing again in PBS (3×), coverslips were rinsed briefly in water and mounted using Prolong Gold (Invitrogen). Microscopy was performed using Olympus AX70 microscope and images were obtained using QCapturePro software (Version 5.1.1.14). Image analysis was performed using Image J (1.45).

2.2.9 – Slot blot

5×10⁶ parasites were resuspended at a final concentration of 5×10⁵ cells/ml in PBS. 100 µl were adhered to nitrocellulose membranes using Mini-fold II Slot-Blot System (Schleicher & Schuell, Keane, NH). The membrane was incubated in 2% powdered non-fat milk for 1 h, then with mouse anti-dsRNA J2 antibody (1:2000) and polyclonal rabbit anti-histone H2A (1:2000; Wong and Beverley, in preparation) in 2% milk plus 0.2% Tween 20 for 1 h. The membrane was washed in 1×PBS plus 0.1% Tween 20 (PBS-T) 3× and incubated in goat anti-mouse IRDye 800 and goat anti-rabbit IRDye 680 (1:10000 each, Licor Biosciences, Lincoln, NE) for 1 h. The membrane was washed 3× in PBS-T and once in 1×PBS. Analysis was performed using the Odyssey Infrared Imaging System and Application Software Version 3.0.16 (LiCor Biosciences). The cut-off point was calculated as 3 standard deviations (S.D.) above the mean absorbance of the LRV negative control.

2.2.10 – ELISA

Stationary phase promastigotes (10⁸ parasites/ml) were lysed in PBS+0.5% Nonidet P40 (NP40). 20 µg of total proteins, equating to approx. 5×10⁶ parasites (as quantified with BCA assay) were adhered to a 96 well plate (Thermo Scientific), which had been pre-coated with poly-lysine (Sigma), overnight at 4°C. After 4 washes in PBS 0.05% Tween20 (PBS-Tw20), lysates were blocked in assay diluent solution (eBioscience) for 2 h at RT, washed again in PBS-Tw20, and incubated for 1 h at 37°C with the primary mouse monoclonal anti-dsRNA J2
antibody (1:2000, English & Scientific Consulting). After 4 more washing steps, a secondary anti-mouse IgG HRP conjugated antibody (1:2500, Promega) was added for 1 h at 37°C. Wells were then washed and dsRNA could be colorimetrically quantified by the addition of o-Phenylenediamine dihydrochloride (OPD) in a phosphate citrate buffer (Sigma). The reaction was stopped by acidification with 0.5 M H₂SO₄ and measured at 490 nm with a Biotek Synergy HT spectrophotometer. The cut-off point was calculated as 3 standard deviations (S.D.) above the mean absorbance of the LRV negative control.

2.2.11 – Dot blot

Stationary phase promastigote pellets were resuspended in 1×PBS, and a small amount was lysed for BCA quantification in 0.5% NP40. Parasite samples in PBS were then adjusted to 0.1 µg/µl of total protein and spotted onto a nitrocellulose membrane using a range of 0.5 to 4 µg of protein per spot (corresponding to approx. 10⁵ to 10⁶ parasites). To test the sensitivity of the method, live parasites were counted, serially diluted between a range of 10 to 1000 parasites and directly spotted on the nitrocellulose membrane. The membranes were then dried before revelation by immunodetection as described above (see “Anti-capsid antibody production and immunoblotting” section), using an anti-dsRNA J2 primary antibody (1:1000, English & Scientific Consulting) and an anti-mouse IgG HRP conjugated secondary antibody (1:2500, Promega).

2.2.12 – Mouse infection and RNA extraction from leishmaniasis lesions

One million stationary phase Lg M4147 LRV<sup>high</sup> or Lg M4147 LRV<sup>neg</sup> promastigotes were injected subcutaneously into the base of the hind footpad of C57BL/6 mice. Lesions were isolated at the peak of infection (approx. 4 weeks post-infection) and homogenized with a mortar and a pestle in PBS. After an initial centrifugation step to remove large debris (50 g for 2 min),
cell supernatant was centrifuged again (600 g for 8 min) and the pellet was directly resuspended in Trizol for total RNA extraction (as described in “qRT-PCR” section). Approximately 50 µg of RNA was obtained from each lesion (40–50 mg) and diluted in water for dot blot analysis with the J2 antibody (see “Dot blot” section).

2.3 – Results

In order to characterize the presence and burden of LRV in L. (Viannia) parasite strains via different methods, we first tested four parasite isolates of varying LRV content [14]. Two clones derived from the L. guyanensis M4147 strain were used: Lg M4147 LRV<sup>high</sup>, known to have a high burden of LRV and Lg M4147 LRV<sup>neg</sup> in which LRV is undetectable by RT-PCR tests [25]. In addition, we also tested two human isolates of L. guyanensis: Lg 1398, derived from a metastatic lesion and known to bear high levels of LRV and Lg 1881, from a CL patient and in which LRV is present at a very low level (at least 10'000 fold less [14]). To best compare the various LRV detection techniques, each was performed on material from a single sample preparation (except for the slot blot). The data shown are representative of the trend gleaned from several independent experiments.

2.3.1 – LRV detection by gel electrophoresis and quantitative real-time PCR

As a starting point, LRV content was estimated using two previously used methods [14]. Firstly, total nucleic acids were extracted from promastigote cultures and analyzed by agarose gel electrophoresis. Here, a 5.3 kb band corresponding to the size of the viral dsRNA genome was detectable in Lg M4147 LRV<sup>high</sup> and Lg 1398 extracts, which was weaker in the latter (Figure 1A, upper panel). This band could be seen more clearly when parasite genomic DNA was eliminated by DNase treatment (Figure 1A, lower panel). As expected, LRV dsRNA was not
detectable in \( Lg \text{ M4147 LRV}^{\text{neg}} \) or in the \( Lg \text{ LRV}^{\text{low}} \) strain \( Lg \text{ 1881} \). Using a serial dilution of nucleic acids from LRV-infected parasites, we estimated that the amount of LRV dsRNA was approximately three to four times higher in \( Lg \text{ M4147 LRV}^{\text{high}} \) than in \( Lg \text{ 1398} \) (Figure 1B).

We then quantified LRV transcript levels, after RNA extraction followed by cDNA synthesis, using two different primer sets that we have already successfully used for LRV detection in \( Lg\text{M4147} \) and \( Lg \text{ M5313} \) strains and their clonal derivatives: SetA, which amplified a 124 nucleotide fragment on the 5′-end of the viral RNA (nucleotide 153 to 277 of the LRV1-4 sequence) [14], and SetB, which amplified a 103-nucleotide fragment in the \( RdRp \) open reading frame (nucleotide 3591 to 3694 of LRV1-4). Quantitative RT-PCR was performed and normalized to both the amplification obtained from the conserved \( kmp11 \) housekeeping gene and the signal obtained from \( Lg \text{ M4147 LRV}^{\text{high}} \). With the SetA primers, \( Lg \text{ 1398} \) showed nearly half the LRV transcripts than \( Lg \text{ M4147 LRV}^{\text{high}} \), while the \( Lg \text{ M4147 LRV}^{\text{neg}} \) line and \( Lg \text{ 1881} \) showed no detectable LRV product. Notable is that no product was obtained with the SetB primers from \( Lg \text{ 1398} \) despite having high levels of LRV (Figure 1B vs Figure 1C). Preliminary sequencing data of the \( Lg \text{ 1398} \) LRV \( RdRp \) open reading frame explained this negative result (H. Zangger, unpublished data), and illustrated a potential problem of a PCR-based approach for LRV screening in uncharacterized parasites.

2.3.2 – \textit{LRV detection by a capsid-specific antibody}

Detection of LRV can also be performed \textit{via} the recognition of viral proteins [27]. A high-affinity rabbit polyclonal antibody (g018D53) was raised against the capsid polypeptide of \( Lg \text{ M5313 LRV} (>98\% \text{ identical to } Lg \text{ M4147 LRV1-4, Genbank accession number: JX313126}) \) and then tested on control strains by immunoblotting and fluorescence microscopy. With both techniques, LRV detection was achieved in \( Lg \text{ M5313} \) (and its derivative
LRV\textsuperscript{high} clones, \textit{Lg} 13 and \textit{Lg} 21; data not shown) as well as in \textit{Lg} M4147 LRV\textsuperscript{high}, showing a strong staining throughout most of the cytosol of promastigotes (Figures 2A and 2B). As expected, no staining was visible in \textit{Lg} 17 (LRV\textsuperscript{low} derivative clone of \textit{Lg} M5313), \textit{Lg} M4147 LRV\textsuperscript{neg} and \textit{Lg} 1881, but neither in the LRV-infected human isolate \textit{Lg} 1398 which is probably due to LRV sequence diversity. Partial \textit{Lg} 1398 LRV sequencing was performed and surprisingly revealed a high identity of its capsid as compared to \textit{Lg} M4147 throughout the entire open reading frame (91\% identical residues, Genbank accession number: JX313127). Epitopes mapping using a 20-mer peptide arrays representing the complete \textit{Lg} M4147 LRV capsid sequence showed that g018D53 recognized uniquely \textit{Lg} M5313 LRV C-terminal capsid sequence, which is poorly conserved in \textit{Lg} 1398, thus explaining why it is not recognized by g018D53 in this strain (Figure 2C and 2D).

2.3.3 – Immunodetection of LRV by a dsRNA-specific antibody

The J2 monoclonal mouse antibody directed against dsRNA allows the detection of various dsRNA viruses independently of their sequences [28], [29]. To gauge its utility for LRV detection, it was first tested on control parasites by fluorescent microscopy using two different fixation protocols (Figures 3A and 3B). For both protocols the staining pattern with the J2 antibody was similar to that seen with the anti-capsid antibody (Figure 2B). Interestingly, a signal was obtained with the strain \textit{Lg} 1398, suggesting that the anti-dsRNA antibody was not limited by differences in sequence amongst LRVs as noted earlier in the qRT-PCR and anti-capsid studies.

From the images acquired via the second protocol (Figure 3B), histograms were constructed to show the distribution of signal intensity between individual cells (Figure 3C). A distinct peak was seen in the \textit{Lg} M4147 LRV\textsuperscript{high} line that was quite separated from that obtained
with the LRV\textsuperscript{neg} line or controls (Figure 3C). The spread of the \textit{Lg} M4147 LRV\textsuperscript{high} peak was somewhat broader than might have been anticipated for a homogeneous population, suggesting some heterogeneity in LRV levels may exist. Similar results have been obtained with anti-capsid antisera (FMK and SMB, not shown).

We also tested the use of a slot-blot technique for estimating LRV load. In this protocol, cells were ‘slotted’ onto nitrocellulose membranes and reacted with J2 to detect dsRNA and anti-histone H2A to control for parasite numbers. Clear differences in LRV\textsuperscript{high} and LRV\textsuperscript{neg} parasites were again observed (Figures 4A and 4B). Both logarithmic and stationary cells were tested showing that the dsRNA signal intensity does not significantly change during culture of the parasite.

The results obtained in IFM or ‘slot’ blotting prompted us to explore more rapid and simple protocols for the use of the J2 anti-dsRNA antibody that may be suitable for screening of field isolates, where sequence divergence amongst LRVs is expected. It was thus transferred to the other immunodetection techniques of ELISA and dot blot. The J2 ELISA method used crude parasite lysate (NP40); it allowed relative quantitation of LRV and confirmation that it was approximately four times more abundant in \textit{Lg} M4147 LRV\textsuperscript{high} than in \textit{Lg} 1398 (Figure 5A). However a clear limitation of this approach is the requirement for high LRV load as illustrated here with a relatively low signal obtained with the \textit{Lg} 1398 strain in comparison to LRV-low/negative strains.

Dot blot tests were performed with whole live parasites spotted directly on nitrocellulose membranes. Distinction between infected or non-infected promastigotes was remarkably reliable (Figure 5B), permitting a relative quantification that reproduced the difference in LRV load between \textit{Lg} M4147 LRV\textsuperscript{high} and \textit{Lg} 1398 (Figures 1B and 5A). In addition to being a simple
technique that is independent of LRV sequence, the dot blot had the advantage of only requiring a very low number of parasites as shown in Figure 5C. Here, LRV could be detected in less than a hundred parasites from the \( Lg \) M4147 \( \text{LRV}^{\text{high}} \) line.

2.3.4 – Screening for LRV infection in human isolates

To assess the applicability of our anti-dsRNA dot blot on field isolates, we used it for LRV screening in human isolates from another \( \text{Leishmania} \) species that had been previously typed and catalogued as LRV positive [24]. Five strains were screened, corresponding to \( L. \) braziliensis isolated from human lesions (Figure 6). As expected, we were able to confirm LRV presence in these isolates. This study suggested that the dot blot method using J2 was a valid approach that can be extended to clinical \( \text{Leishmania} \) isolates from human biopsy.

2.3.5 – Screening for LRV presence in \( L. \) braziliensis isolated from an infected patient

To demonstrate that our anti-dsRNA immunodetection approach may be a relevant diagnostic tool in a clinical setting, it was tested on freshly isolated \( \text{Leishmania} \) parasites obtained from an \( L. \) braziliensis infected patient. The subject contracted leishmaniasis in Bolivia, which was later typed by PCR as being \( L. \) braziliensis (data not shown). Two parasite samples were taken: \( Lb \)2169 and \( Lb \) 2192, derived respectively from a primary cutaneous lesion before treatment, and a secondary/metastatic lesion appearing some time after treatment had started. Parasites from these biopsies were cultivated and directly tested for LRV presence by dot blot using the anti-dsRNA antibody as described above. \( Lg \) M4147 \( \text{LRV}^{\text{high}} \) and \( Lg \) M4147 \( \text{LRV}^{\text{neg}} \) parasites were used as positive and negative controls respectively. As shown in Figure 7A, a clear signal, although weaker than for \( Lg \) M4147 \( \text{LRV}^{\text{high}} \), was detected in both parasite isolates from this infected patient. To ascertain that this positive signal was genuinely due to the presence of LRV, the samples were retested using some of the other LRV detection techniques,
i.e. immunofluorescence microscopy (Figure 7B) and isolation of viral dsRNA, with clear
detection of a ssRNase- and DNase-resistant 5.3 kb band (Figure 7C). Sequencing of this newly
identified LRV is currently in progress. Because the presence of LRV may be an aggravating
factor in the development of refractory metastatic disease, early diagnosis of LRV content may
aid diagnosis and be used to guide treatment strategies. This experiment demonstrated the ease
and accuracy of dsRNA detection and reinforced the broad applicability of the anti-dsRNA
antibody in the detection of LRV across Leishmania species.

2.4 – Discussion

The presence of LRV in Leishmania (Viannia) species is suspected to be a major
aggravating factor in the dissemination and persistence of leishmaniasis. Therefore, the detection
of LRV might prove clinically beneficial, guiding treatment or providing prognostic information.
In this study, we evaluated several approaches of LRV detection, starting with the identification
of a 5.3 kb viral dsRNA band in total parasitic nucleic acid (Figure 1A) [14]. This method,
however, had the marked disadvantage of requiring at least 10^8 parasites and a high LRV load.
On the contrary, the qRT-PCR approach is both highly sensitive as well as quantitative but its
use as a first line diagnostic could be limited in the field in case of LRV genetic polymorphism
(as illustrated with the SetB primers in Figure 1C). Further work would be required to identify
the highly conserved regions amongst all LRV genomes in divergent parasite strains in order to
avoid such a problem. Immunodetection by anti-LRV antibodies also proved to be clinically
applicable with the advantage of qualitative analysis by fluorescence microscopy, revealing an
interesting cytosolic clustering of viral particles (Figure 2B). Anti-capsid antibodies, however,
have the same potential limitation as qRT-PCR due to their dependence on the underlying capsid
sequence.
In this report, we describe new sequence-independent LRV detection techniques, using the anti-dsRNA J2 antibody, which could then be used in the field against any LRV strain circumventing the problem of sequence specificity that could occur. It was found to be effective and quantitative in microscopy, slot blot, ELISA and dot blot assays using parasites or lesions extracts, where it detected LRV in all LRV-positive control strains. All the strains analyzed in this study and the results obtained from each method are summarized in Table 1. The anti-dsRNA-based dot blot technique stood out as the candidate method for use in the field, having sufficient sensitivity and ease of use to allow rapid LRV detection at a relatively low cost that could be performed at a large scale in a clinical setting (Table 1).

In our previous analysis [14], we showed that the metastatic parasites in the Golden hamster model as well as a human ML isolate were positive for LRV, while non-metastatic and a human CL-derived strain were negative or very poorly infected. From the analysis reported here, we could detect the presence of LRV in other Leishmania isolates, including again L. guyanensis, but in addition in freshly isolated L. braziliensis parasites from human lesions. Finally, we showed that LRV could also be detected directly from minute lesion biopsies in mice thus avoiding parasite isolation and promastigote cultivation, which is a clear advantage when adapting of the technique such a diagnostic technique for field applicability. We propose that this approach could now be finalized for use on a mass-scale to determine the prevalence of LRV in L. (Viannia). This would greatly aid in confirming the correlation between LRV presence and clinical phenotype. If a significant trend is established, LRV detection could be used as a prognostic tool, perhaps guiding treatment strategies to prevent the metastatic complications often observed in some Leishmania (Viannia) infected patients.
Acknowledgments

The authors are grateful to J. Patterson and Y.T. Ro for providing the parental lines from which the clonal luciferase-expressing LRV$^{\text{high}}$ or LRV$^{\text{neg}}$ Lg M4147 strain used here were derived, as well as anti-LRV M4147 capsid antisera. We also thank Natacha Cossy, Tugce Tekozgen, Leyder Lozano and Tatiana Proust for technical help, Catarina Servis and the Protein and Peptide Chemistry Facility (University of Lausanne), Florence Morgenthaler from the Cellular Imaging Facility (CIF) of Lausanne University in Epalinges for microscopy imaging and Habib Zalila for the input on graphics.

2.6 – References


### Table 2-1: LRV status of the analyzed strains according to detection method.

<table>
<thead>
<tr>
<th>LRV load</th>
<th>Strain</th>
<th>dsRNA extraction</th>
<th>qRT-PCR</th>
<th>WB + IFM (capsid Ab)</th>
<th>IFM (J2)</th>
<th>SB (J2)</th>
<th>ELISA (J2)</th>
<th>DB (J2)</th>
</tr>
</thead>
<tbody>
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<td>High</td>
<td>Lg M4147 LRV&lt;sub&gt;high&lt;/sub&gt;</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>Lg 1398</td>
<td>+&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+</td>
<td>+&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+</td>
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<td></td>
<td>+</td>
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<tr>
<td>Low</td>
<td>Lg 1881</td>
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<td>–&lt;sup&gt;a&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
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<tr>
<td></td>
<td>Lg 03</td>
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<td>–&lt;sup&gt;a&lt;/sup&gt;</td>
<td>–</td>
<td>–&lt;sup&gt;a&lt;/sup&gt;</td>
<td>–&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>–</td>
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</tbody>
</table>

<sup>a</sup>As shown in Ives et al., 2011; by qRT-PCR analysis, Lg 1881, Lg 03 and Lg 17 were classified as LRV<sub>low</sub> harboring at least 10,000 fold less viral transcripts than the highly infected strains.

<sup>b</sup>As shown in Salinas et al., 1996.

<sup>c</sup>Performed in this study but not shown in the figures.

<sup>d</sup>Only with specific primers.

Abbreviations: WB = western blotting/IFM = immunofluorescence microscopy/ SB = slot blot/DB = dot blot.

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2.8 – Figures

Figure 2-1: Detection of LRV in nucleic acid extracts.

A and B. Visualization of viral genomic dsRNA by gel electrophoresis. A. Total nucleic acid from stationary phase promastigotes was treated with ssRNase then migrated in a 1% agarose gel. The sample was either kept intact (1 mg, upper panel) or digested with RQ-DNase (5 mg, lower panel). B. To quantify viral dsRNA in Lg 1398 relative to Lg M4147 LRV\textsuperscript{high}, various concentrations of nucleic acid (2, 1 and 0.5 mg) were digested with RQ-DNase and migrated as above. C. Quantification of LRV transcript by qRT-PCR. Total parasitic and viral cDNA was prepared for qRT-PCR and amplified using primers specific for LRV (SetA and SetB, see material and methods for sequences). Viral transcript was quantified as normalized to the parasitic housekeeping gene kmp11 then adjusted relative to Lg M4147 LRV\textsuperscript{high}.
Figure 2-2: Detection of LRV with a polyclonal anti-capsid antibody (g018d53) and epitope mapping.

A. Western blot. Total parasitic protein extract (40 mg) was separated on a 10% acrylamide denaturing gel then transferred onto a nitrocellulose membrane where the LRV capsid could be detected using the rabbit polyclonal antibody g018d53 (upper panel). A Ponceau staining of the same membrane shows total parasitic protein (lower panel). B. Immunofluorescence microscopy. Red: capsid (g018d53 Ab). Blue: DAPI integrated into kinetoplast and nuclear DNA. Capsid immunofluorescence was visualized with a standardized exposure time in all images. C. 74 overlapping peptides (20-mer) covering the complete
sequence of Lg M4147 LRV1-4 capsid were spotted on a cellulose membrane (30 peptides per lane as indicated) and incubated with the g018d53 antibody to identify the recognized epitopes. D. Sequence alignment of the LRV capsids from Lg M4147, Lg M5313 and Lg 1398 in the C-terminal region covering the epitopes recognized by the g018d53 antibody (shown in C). The residues that are not identical to the Lg M5313 LRV sequence are highlighted in a black box.
Figure 2-3: Detection of LRV with a monoclonal anti-dsRNA (J2) antibody by immunofluorescence microscopy.

A. Reference strain analysis (protocol A, see “Material and methods”). Green: dsRNA (J2 Ab). Blue: DAPI (standardized exposure time in all images). B. Phase and immunofluorescent images of Lg M4147 LRV\textsuperscript{high} or LRV\textsuperscript{neg} cells were obtained in the presence or absence of J2 antibody (protocol B). C. Quantitative immunofluorescence (protocol B). The fluorescent intensity per cell was assessed using Image J software on Lg M4147 LRV\textsuperscript{high} or LRV\textsuperscript{neg} cells following IFM with the J2 antibody. Cells from phase images were identified and the fluorescent intensity average over the area of the cell was recorded. 108–160 cells from 2 distinct fields were measured, and histogram plots were made using Excel software.
LRV^{high}, no primary antibody (square symbol, dashed line); LRV^{high} with J2 (square symbol, solid line); LRV^{neg}, no primary antibody (circle symbol, dashed line); LRV^{neg} with J2 (circle symbol, solid line).
Figure 2-4: Detection of LRV using slot blots and J2 antibody.

A. $5610^4$ parasites were blotted onto nitrocellulose membranes and incubated with J2 or anti-histone H2A antibodies. B. Quantification of the signal intensity for cells in logarithmic or stationary growth phase: dsRNA signal was quantified relative to the histone H2A signal. The cut-off line was calculated as 3 standard deviations (SD) above the mean absorbance of the LRV- negative that showed the highest value (log phase).
Figure 2-5: Detection of LRV in total parasite lysate using J2 antibody.

A. ELISA. Total lysates from $5 \times 10^6$ promastigotes were coated on 96 wells plates and dsRNA was quantified colorimetrically at 490 nm relative to Lg M4147 LRV$^{\text{high}}$ after background subtraction (uncoated control wells). The cut-off line was calculated as 3 standard deviations (SD) above the mean absorbance of the LRV-negative that showed the highest value (Lg 1881). B. Dot blot. $10^5$ to $5 \times 10^5$ promastigotes were spread directly onto a nitrocellulose membrane and dsRNA was detected using the J2 antibody (upper panel). A Ponceau stain of the membrane shows total protein concentration was similar across samples (lower panel). C. Dot blot sensitivity screening. A dot blot was performed in a serial dilution of 1000 to 10 parasites from LRV-positive and negative control strains (Lg M4147 LRV$^{\text{high}}$ and Lg M4147 LRV$^{\text{neg}}$).
Figure 2-6: Screening for LRV in human isolates of Leishmania.

Parasites of 5 different *L. braziliensis* strains previously shown to harbor LRV [24] were analyzed by dot blot (1 to 4 mg total protein/spot).
Figure 2-7: Screening for LRV in freshly-isolated human L. braziliensis.

A. Dot blot analysis of two parasite samples obtained from separate lesion biopsies in an infected patient: Lb 2169 and Lb 2192. Live parasites (1 to 4 mg total proteins) were spotted on a nitrocellulose membrane for LRV dsRNA detection by dot blot (J2 antibody). Lg M4147 LRV$^{\text{high}}$ and LRV$^{\text{neg}}$ were used as positive and negative controls. Upper panel: dsRNA detection by dot blot (J2). Lower panel: verification of protein quantity by Ponceau staining. B. J2 anti-dsRNA analysis of Lb 2169 by fluorescence microscopy. Green: dsRNA (J2 Ab). Blue: DAPI. C. Isolation of viral genomic dsRNA from the Lb 2169 strain. Intact and DNase-digested total nucleic acids from Lb 2169 parasites and Lg M4147 LRV$^{\text{high}}$ as a control, were analyzed by gel electrophoresis (similarly to Figure 1A). Note: with high resolution gels such as presented here (in contrast to Figure 1), the viral genome often appears as a doublet.
Table: Detection of LRV in mice footpad lesions.

<table>
<thead>
<tr>
<th>Promastigotes</th>
<th>Lesions</th>
</tr>
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<tbody>
<tr>
<td>LRV\textsuperscript{high}</td>
<td>LRV\textsuperscript{neg}</td>
</tr>
<tr>
<td>200 ng</td>
<td>400 ng</td>
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<td>200 ng</td>
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<td>25 ng</td>
<td>25 ng</td>
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<tr>
<td>10 ng</td>
<td>5 ng</td>
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</table>

*Figure 2-8: Detection of LRV in mice footpad lesions.*

Dot blot analysis on total RNA extracted from mice lesions infected with Lg M4147 LRV\textsuperscript{high} and Lg M4147 LRV\textsuperscript{neg}. Whole parasite (‘total’) and RNA extracts from Lg M4147 promastigotes were also loaded as a control. The amount of protein and RNA loaded is indicated on the left and right side of the figure respectively.
Chapter 3: Antiviral screening identifies adenosine analogs targeting the endogenous dsRNA *Leishmania* RNA virus 1 (LRV1) pathogenicity factor
Preface


Specifically, JIR created and interpreted the 3D model of the LRV1 RDRP with advice from SMB and FMK. JIR composed the related figure, wrote the caption, and edited the manuscript written by FMK and SMB.

This chapter was previously published:

3.1 – Abstract

The endogenous double stranded RNA virus Leishmaniavirus (LRV1) has been implicated as a pathogenicity factor for leishmaniasis in rodent models and human disease, and associated with drug treatment failures in L. braziliensis and L. guyanensis infections. Thus methods targeting LRV1 could have therapeutic benefit. Here we screened a panel of antivirals for parasite and LRV1 inhibition, focusing on nucleoside analogs to capitalize on the highly active salvage pathways of *Leishmania*, which are purine auxotrophs. Applying a new capsid flow cytometry assay, we identified two 2'-C-methyladenosine (2CMA) analogs showing selective inhibition of LRV1. Treatment resulted in loss of LRV1 with first order kinetics as expected for random virus segregation, and elimination within 6 cell doublings, consistent with a measured LRV1 copy number of about 15. Viral loss was specific to antiviral nucleoside treatment and not induced by growth inhibitors, in contrast to fungal dsRNA viruses. Comparisons of drug-treated LRV1\(^+\) and LRV1\(^-\) lines recapitulated LRV1-dependent pathology and parasite replication in mouse infections, and cytokine secretion in macrophage infections. Agents targeting Totiviridae have not been described previously, nor are there many examples of inhibitors acting against dsRNA viruses more generally. The compounds identified here provide a key proof of principle in support of further studies identifying efficacious antivirals for use in in vivo studies of LRV1-mediated virulence.

3.2 – Introduction

Protozoan parasites of the genus *Leishmania* are responsible for leishmaniasis in many regions of the world, with 12 million current cases (accompained by at least 10-fold more bearing asymptomatic infections), and nearly 1.7 billion people at risk (1-5). The disease has three predominant clinical manifestations, ranging from the relatively mild, self-healing
cutaneous form, to mucocutaneous lesions where parasites metastasize to and cause destruction of mucous membranes of the nose, mouth, and throat, or fatal visceral disease. Disease phenotypes segregate primarily with the infecting species; however, it is not fully understood which parasite factors affect severity and disease manifestations.

One recently identified parasite factor contributing to disease severity in several *Leishmania* species is the RNA virus *Leishmaniavirus* (6, 7). These endobiont viruses classified within the Totiviridae are comprised of a single-segmented dsRNA genome that encodes only a capsid protein and an RNA-dependent RNA polymerase (RDRP) (8, 9). *Leishmaniavirus* is most frequently found in New World parasite species in the subgenus Viannia (as LRV1) such as *L. braziliensis* (*Lbr*) and *L. guyanensis* (*Lgy*), which cause both cutaneous and mucocutaneous disease (6), and is found sporadically in Old World subgenus *Leishmania* species (as LRV2) (10, 11). Mice infected with LRV1-bearing strains of *Lgy* exhibit greater footpad swelling and higher parasite numbers than mice infected with LRV1-negative *Lgy* (7). Similarly, macrophages infected in vitro with LRV1+ *Lgy* or LRV2+ *L.aethiopica* release higher levels of cytokines, which were dependent on Toll-like receptor 3 (7, 10). Recently, methods for systematically eliminating LRV1 by RNA interference have been developed, enabling the generation of isogenic LRV1 negative lines allowing the extension of the LRV1-dependent virulence paradigm to *L. braziliensis* (12).

A key question is the relevancy of the studies carried out in murine models to human disease. For *Lgy*, patients infected with LRV1+ strains show an increased severity of cutaneous disease (13). In humans, *Lbr* is associated with cutaneous leishmaniasis, as well as the larger share of the more debilitating mucocutaneous leishmaniasis (MCL). Thus far there are no data available in humans permitting tests of the association of LRV1 with *Lbr* parasite burden nor the
severity of CL, which can show a range of presentations (14, 15). In lieu of such information, studies have focused on the association of LRV1 with MCL vs CL, which is thought to reflect primarily immunopathology, rather than parasite numbers (2, 6, 14-16). While in some studies LRV1 was not correlated with MCL clinical manifestations (17, 18), in others there was a strong association (6, 19, 20). The basis for these discrepancies is of considerable interest, hypotheses for which include other parasite or host factors known to play a significant role in the development of MCL (13, 21, 22), or microbial sources including the microbiota or coinfections (23). Recent studies show that the presence of LRV1 in clinical isolates of \textit{Lbr} and \textit{Lgy} correlates with drug treatment failure (17, 20), phenomena that could readily be explained by the increased parasite numbers and/or altered host responses predicted from animal models (7, 13, 24). Thus, current data support a role for LRV1 in increasing disease severity in human leishmaniasis (13). This suggests that therapies targeting LRV1 specifically could be applied towards amelioration of disease pathology. As one approach, murine vaccination using the LRV1 capsid results in significant protection against LRV1+ \textit{Lgy} (25).

Here we describe a complementary approach, targeting LRV1 directly using small molecule inhibitors. While effective antivirals are available for many viral targets including retroviruses, DNA viruses and ssRNA viruses (26), little effort has gone into agents acting against dsRNA viruses (27). These comprise at least ten viral families (Birnaviridae, Botybirnaviridae, Chrysoviridae, Cystoviridae, Megabirnaviridae, Partitiviridae, Picobirnaviridae, Quadriviridae, Reoviridae and Totiviridae), infecting a wide array of hosts including fungi, plants and animals (28). Some constitute important agricultural pathogens while rotaviruses (Reoviridae) cause serious human disease. For protozoan viruses, their role in the exacerbation of human disease is only now beginning to be appreciated (6, 29). Since viral
elements are critical factors acting to exacerbate the disease where studied, candidate anti-LRV1 agents should be viewed as ‘anti-pathogenicity’ treatments rather than sterilizing cures (30), which could be used alone or more likely in combination with existing anti-leishmanial agents in the treatment of ongoing infection.

As a starting point we focused on nucleoside analogs, a class which includes many widely used and effective antivirals (Table S1) (26). Following uptake and activation to the triphosphate form, these primarily target viral replication, with different classes acting preferentially against viral DNA or RNA polymerases (RDRP) or reverse transcriptases, as well as cellular metabolism. A second rationale was that *Leishmania* are purine auxotrophs, with highly active and multiply redundant pathways for uptake and activation of nucleobases and nucleosides (31). Indeed, a great deal of prior effort has been devoted to the development of anti-leishmanial nucleoside analogs; however, while allopurinol is commonly used as a veterinary agent, it has proven more difficult to find agents of sufficient potency and selectivity against *Leishmania* to be used widely against human leishmaniasis (32). We reasoned that the highly divergent properties of Totiviridae RDRPs, relative to the polymerases of both the *Leishmania* and mammalian hosts (as well as other viral RDRPs), could prove fertile grounds for antiviral discovery, especially when coupled with potentiation by the parasite’s powerful nucleoside/base salvage pathways.

3.3- Methods

3.3.1 – Parasites and growth media.

Most studies were performed using luciferase-expressing transfectants of *L. guyanensis* (MHOM/BR/78/M4147) described previously (LRV1\(^+\) LgyM4147/SSU:IR2SAT-LUC(b)c3 and LRV\(^-\) LgyM4147/pX63HYG/SSU:IR2SAT-LUC(b)c4 (54)); these lines are termed LgyLRV1\(^+\)
and LgYL RV1\textsuperscript{-} respectively. Two strains of LRV1\textsuperscript{+} \textit{L. braziliensis} were examined, LEM2780 (MHOM/BO/90/CS) and LEM3874 (MHOM/BO/99/IMT252 n\textsuperscript{3}) (12). Parasites were grown in Schneider’s media (Sigma, St. Louis, MO) prepared according to the supplier’s instructions with pH adjusted to 6.5 and supplemented with 0.76 mM hemin, 2 μg/ml biopterin, 50 U/ml penicillin, and 50 μg/ml streptomycin, and 10% heat inactivated fetal bovine serum. Cell concentrations were determined using a Coulter Counter (Becton Dickinson).

### 3.3.2 – Drug inhibition tests.

Compounds were purchased or obtained as summarized in Table S1, and the structure of the two most active anti-LRV1 compounds are shown in Fig. S1. Stock solutions were prepared as recommended by the source, typically in DMSO at 50 mM, and tested against parasites at 100 μM or the maximum concentration permitted by drug solubility (Table S2). Parasites were inoculated at 2 x 10\textsuperscript{5} cells/ml into Schneider’s media lacking supplemental adenine. Growth was evaluated after 2 days, prior to the controls reaching stationary phase growth, at which time parasite numbers had increased nearly 100 fold. Experiments were performed in sets of 10 test compounds, along with LRV1\textsuperscript{+} and negative controls; the agreement amongst independent experiments amongst the controls was excellent, and the results are shown averaged together across all experiments (Table S2).

### 3.3.3 – LRV1 capsid flow cytometry.

10\textsuperscript{7} cells were fixed at room temperature (RT) using 2% paraformaldehyde (Thermo Fisher) in PBS for 2 minutes. They were then incubated in blocking buffer (10% normal goat serum (Vector Laboratories) and 0.2% Triton X-100 in PBS) for 30 min at RT. Anti-Lgy LRV1 capsid antibody (35) was added (1:20,000 dilution) and incubated at RT for 1 hr. After two washes with PBS, cells were resuspended in in 200 ul PBS with Alexa488-labeled goat anti-
rabbit IgG (Alexafluor, Invitrogen, Eugene, OR, 1:1,000; or Thermo Fisher, 1:2,000 dilution),
and incubated 1 hr at RT. After two additional washes with PBS, cells were subjected to flow
cytometry, gating for single cells using forward and side scatter and the data analyzed using
CellQuest® software (BD Bioscience).

3.3.4 – RNA purification, cDNA preparation, and qRT-PCR.

10^7 cells were resuspended in 350 μl TRIzol® Reagent and RNA was extracted using the
Direct-zol RNA purification kit according to protocol (Zymo Research). RNA was then treated
with DNase I (Ambion) for 1 hour at 37°C and re-purified using RCC-5 column purification
(Zymo Research). cDNA was prepared using Superscript III (Invitrogen) and random priming
according to protocol. RNA denaturation occurred at 65°C for 5 min. RT-PCR tests were
performed using LRV1-specific primers (SMB4647 5’-TBRTWGRCACAGTGAYGAAGG
and SMB4648 5’CWACCCARWACCABGGBGCCAT) or β-tubulin mRNA (SMB5023 5’-
AAGCTATATAAGTATCAGTTTCTGTACTTTA and SMB2110 5’-
GACAGATCTCATCAAGCACGGAGTGCATCAAGC). Quantitative RT-PCR (qRT-PCR) was
performed as previously described (36), with a 123 bp fragment of LRV1 capsid mRNA
amplified with primers SMB5335 (5’-CTGACTGGACGGGGTAAT) and SMB5336 (5’-
CAAAAACACTCCCTTACGC), and a 100 bp fragment of KMP-11 (a Leishmania housekeeping
gene) with primers SMB5548 (5’-GCCTGGATGAGGAGTCAACA) and SMB5549 (5’-
GTGCTCCTTCATCTCGG). The reaction utilized Power SYBR® Green (Applied Biosystems)
in an ABI Prism 7000. Initial denaturation was at 95°C for 10min followed by 40 cycles of
amplification with 15s at 95°C, and 1 min at 60°C. Data was analyzed using ABI 7000 SDS
software (version 1.2.3) and normalized using ΔΔCT method (60). RNA slot blot analysis was
performed as described (36). The LRV1 copy number per cell was estimated in comparison to a
standard curve generated using DNA from a plasmid bearing the LRV1 capsid gene (B6760, pBSKLRV1-4) and the average yield of RNA/cell across multiple Lgy RNA preparations (5.12 ±1.17 μg/10⁷ cells; n = 34).

3.3.5 – Isolation of LRV1⁺ and LRV1⁻ clonal lines by brief treatment with 2CMA.

LgyLRV1⁺ parasites were grown for one passage in media containing 25 μg/ml nourseothricin (Werner BioAgent, Jena, Germany) to verify the presence of the integrated LUC gene (54). Cells were then grown 1 passage in the absence of nourseothricin, and inoculated into Schneider’s media at a concentration of 2 x 10⁵ cells/ml into media containing 10 μM 2CMA. Growth was measured and LRV1 quantitated by capsid flow cytometry. At various times, cells were either plated directly, or transferred to drug-free media, and allowed to grow an additional 6 cell doublings prior to plating. For both, the semisolid M199 media contained 50 μg/ml nourseothricin, and cells were diluted so that no more than ~100 colonies were obtained per plate.

3.3.6 – Macrophage infections, cytokine assays, and mouse infection.

Infections of C57BL/6 mouse bone marrow derived macrophages (BMDM) and cytokine assays were performed as previously described (7, 10). Poly I:C was obtained from Invivogen and used at 2 μg/ml. For mouse infections, 5 to 6 week old C57BL/6 mice were purchased from Jackson Laboratories (United States). Parasites were grown into stationary phase (2 full days) and 10⁶ parasites were injected on the plantar aspect of the left foot. Measurement of footpad swelling was performed weekly using a Vernier caliper. Parasite numbers were assessed by luminescence of an integrated firefly luciferase reporter, measured using an IVIS 100 instrument as described previously (7, 54) and analyzed with Living Image software version 2.60 (Perkin Elmer, Waltham, MA).
3.3.7 – Statement identifying institutional and/or licensing committee approving animal experiments.

Animal handling and experimental procedures were undertaken with strict adherence to ethical guidelines relevant in both host countries. These are set out by the SFVO and under inspection by the Department of Security and Environment of the State of Vaud, Switzerland. Experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the United States National Institutes of Health. Animal studies were approved by the Animal Studies Committee at Washington University (protocol #20090086) in accordance with the Office of Laboratory Animal Welfare's guidelines and the Association for Assessment and Accreditation of Laboratory Animal Care International.

3.4 – Results

3.4.1 – Measurement of LRV1 levels by capsid flow cytometry.

As LRV1 (like most Totiviridae) is not shed from the cell (33, 34), we developed a flow cytometric assay to measure intracellular LRV1 capsid levels on a per-cell basis. To detect LRV1 we used binding to a rabbit anti-LgyLRV1 capsid antiserum (35) followed by detection with AlexaFluor488-conjugated goat anti-rabbit IgG. We found that fixation with 2% paraformaldehyde followed by permeabilization with Triton X-100 yielded a clear LRV1-dependent profile (Fig 1A). Titration of the anti-capsid antiserum showed that dilutions around 1:16,000 gave a strong signal with excellent selectivity between LgyLRV1+ and LRV1− (Fig. 1B), with little background staining evident in immunofluorescence microscopy (not shown). Under these conditions and as seen in previous immunofluorescence studies (36), LgyLRV1+
showed a strong, homogeneous LRV1 distribution (Fig. 1A). We attempted similar studies with anti-dsRNA antibodies (36), but were unable to identify fixation conditions that gave similarly clear discrimination between \( \text{Lgy}_{\text{LRV1}}^+ \) and \( \text{LRV1}^- \) by flow cytometry.

3.4.2 – Inhibition tests.

We acquired a collection of 81 compounds, primarily nucleoside or nucleobase analogs, including ones shown previously to be active against diverse viruses, tumor cells, or \textit{Leishmania} (Table S1, S2; Fig. S1). These were examined for their ability to inhibit the growth of \( \text{Lgy}_{\text{LRV1}}^+ \) and virus levels by LRV1 capsid flow cytometry. \( \text{Lgy}_{\text{LRV1}}^- \) parasites grew similarly to \( \text{Lgy}_{\text{LRV1}}^+ \), and were used as virus-negative controls. These data revealed three patterns (Fig. 2). For most compounds, LRV1 capsid levels were not significantly affected, within a factor of \( \sim 3 \) (Figs. 2, black or red dots within large dashed gray and red circles; Table S2, Fig. S2). All nucleobase analogs fell within this group, as did foscarnet (a structure analog of pyrophosphate). Within this group, a subset showed more than 10-fold inhibition of \( \text{Lgy} \) growth (Fig. 2, red circle; Table S2, Fig. S3a/b), including known anti-leishmanials such as allopurinol, mycophenolic acid and APP. Several additional compounds showed leishmanial inhibition at the concentration tested (Table S1,S2, Figs 2, S2A), however these were deprioritized for various reasons including known mammalian cell toxicity. In the initial screens several compounds showed modest elevation of LRV1, often accompanied by growth inhibition (Figs. 2, S2A; Table S2). However, this effect was not always reproducible and was not pursued further.

Two compounds strongly reduced LRV1 capsid levels with minimal impact on parasite growth (Fig. 2, green circle; Fig. S1, Table S2). \( 2'\)-C-methyladenosine (2CMA) and \( 7\text{-deaza-}2'\)-\( \text{C-methyladenosine (7d2CMA) resulted in 12- fold reductions in LRV1 capsid levels, showing 30% and 90% inhibition of parasite density respectively when tested at 100 \mu\text{M}. Both had
previously been shown to inhibit the HCV RDRP following activation (37, 38). In contrast, 2’-C-methylcytidine or guanosine had little effect on LRV1 levels or Lgy growth (Fig.2, blue dots). Compounds bearing a variety of other 2’ modifications (alone or in combination, with various bases) showed little effect on LRV1. These included sofosbuvir and mericitabine (related to 2’-C-methyl-2’-F uridine or cytidine respectively), both of which show strong activity against HCV (39, 40), or NITD008, which shows good activity against flaviviruses (41). These data suggest a strong preference for both the nature of the 2’-C substitution, as well as adenine as the base. Note that these data cannot discriminate between effects arising from direct inhibition of RDRP or other viral processes, nor drug metabolism (phosphorylation and/or resistance to nucleoside hydrolases).

Previously a *Leishmania* cysteine proteinase activity was implicated in the cleavage of the LRV1 capsid-RDRP fusion protein, potentially important for LRV1 biogenesis (42). However, no effects on Lgy growth and only minimal effects on LRV1 capsid levels were observed with three cysteine proteinase inhibitors tested (E64, E64d and CA-074; Table S2), relative to the effects of 2CMA or 7d2CMA.

3.4.3 – 2CMA preferentially inhibits LRV1 replication.

Titrations were performed to quantitate the potency of 2CMA and 7d2CMA against Lgy growth and LRV1, measuring the relative cellular growth rate to better assess fitness effects. For 2CMA, the EC₅₀ was estimated to be ~3 μM for LRV1 capsid inhibition, versus >100 μM for parasite growth (Fig. 3A), at least 30-fold selective. To assess the effects on replication of the dsRNA LRV1 genome directly, we used quantitative anti-dsRNA slot blots (Fig. 3A (36)), which showed an EC₅₀ of ~ 1 μM, slightly less than seen with capsid inhibition and consistent with the anticipated targeting of the RDRP. With 7d2CMA, an EC₅₀ of ~5 μM was seen against LRV1
capsid expression, versus ~ >100 μM for Lgy growth, again with about >20-fold selectivity (Fig. 3B). Several studies were carried out with L. braziliensis strains bearing LRV1 (12). The 2CMA EC₅₀ for LbrLRV1 was similar to that seen with LgyLRV1 (~3 μM), however parasites were somewhat more susceptible to growth inhibition (EC₅₀ 50-100 μM). As the available quantities of 7d2CMA were limiting and both compounds were similarly selective for Lgy, we focused thereafter on 2CMA.

3.4.4 – 2CMA LRV1 inhibition is unaffected by exogenous adenine nor is synergy seen with antileishmanial nucleobases.

We asked whether the 2CMA potency was affected by the presence of exogenous adenine, present at about 5-33 μM in the yeast extract component of Schneider’s medium (43). The addition of exogenous adenine up to 400 μM had no impact on LRV1 inhibition by 100 μM 2CMA nor did it alter LRV1 levels in LgyLRV1⁺ (Fig. S3C). APP (4-aminopyrazolopyrimidine) showed similar inhibition of Lgy growth and LRV1 levels, while at the highest concentration tested, allopurinol inhibited Lgy growth or LRV1 capsid levels by 30 or 60% respectively (Fig. S3A). We then explored potential interactions between 2CMA and antileishmanial nucleobases. However, no change in the EC₅₀ for 2CMA inhibition of Lgy growth or LRV1 capsid synthesis was seen with increasing concentrations of allopurinol (~ 3 μM; Fig. S3D).

3.4.5 – LRV1 inhibition is independent of Leishmania growth inhibition.

Agents inducing stress and/or growth arrest have been employed to cure fungal Totiviridae, with cycloheximide (CHX) used often (44, 45). Growth of Lgy at 10 or 100 nM CHX resulted in an increase in population doubling time, from ~7.7 hr to 11.2 or 44.7 hr respectively, without significant cell death as evidenced by resumption of WT growth following CHX removal (Fig. 4A). Despite the strong effects on growth, LRV1 capsid levels were
unaffected nor was the emergence of a “LRV1-negative” parasite population seen at any CHX concentration (Figs 4B,C). Similar results were obtained with clotrimazole, which inhibits Leishmania growth through inhibition of sterol synthesis (Fig. 4D). Lastly, no correlation was seen between LRV1 levels and growth rate in our test compound screening (Figs. 2, S2) or exposure to hygromycin B (46). Thus, inhibition of *Leishmania* growth alone does not alter LRV1 levels.

3.4.6 – *Viral loss occurs by random dilution.*

The availability of an inhibitor with strong selectivity for LRV1 over parasite growth provided the first opportunity to test the assumption that cytosolic Totiviruses are passed randomly to daughter cells during mitosis (34, 47). For maximal LRV1 inhibition, parasites were inoculated into 100 μM 2CMA, which increased the population doubling time from 6.4 hr to 8.5 (Fig. 3). The average LRV1 levels immediately declined, with capsid and RNA levels falling in parallel (Fig 5A, B). Importantly, when plotted as a function of number of cell divisions, loss of LRV1 capsid and RNA followed a first order linear relationship, with a 50% loss at every doubling (Fig. 5A, B). When visualized at the population level by flow cytometry, LRV1 capsid levels/cell declined homogeneously at every time point tested until only background staining was evident by 6 cell doublings (Fig. 5C). Both of these observations closely match the expectation for the random distribution of LRV1 particles to daughter cells during mitosis and successive cell divisions.

3.4.7 – *2CMA induces LRV1 negative populations.*

To explore the loss of LRV1 further, we performed a series of ‘washout’ experiments, growing *LgyLRV1* in 100 μM 2CMA for 1, 3, 4 or 6 cell doublings followed by transfer to drug free media. After one doubling, a time when LRV1 levels had only decreased 2-fold, LRV1
capsid levels rapidly returned to WT levels and distribution. In contrast, when 2CMA was maintained for 3 or 4 cell doublings, resulting in a homogeneous population showing on average 8- or 16-fold less LRV1 capsid expression, the ‘washout’ lines now showed two distinct populations (Figs. 5C, D). One expressed LRV1 at levels similar to control LgyLRV1\(^+\), while the other resembled LgyLRV1\(^-\) (Fig 5D, first 2 panels). Parasites with LgyLRV1\(^+\) capsid levels were the majority (55%) in the 3 doubling washout population, whereas these had declined to 36% percent in the 4 doubling washout population (Fig 5D). The LgyLRV1\(^-\) population increased from 31 to 50% of the total cell population during this time. Lastly, after six cell doublings of growth with 2CMA, the LRV1 capsid profile was indistinguishable from that of the LgyLRV1\(^-\) and the 6 doubling washout population revealed only parasites maintaining the LgyLRV1\(^-\) capsid staining profile (Fig. 5D). This population was maintained for at least 6 passages (~40 cell doublings) without return of any demonstrable LRV1\(^+\) parasites.

Several conclusions emerge from these studies: first, the effective LRV1 copy number per cell must be relatively low, as otherwise an LRV1\(^-\) negative population could not emerge after only 3-6 cell doublings (Fig. 5), roughly corresponding to copy numbers of 8-64 (\(2^3 - 2^6\)) and consistent with fraction of LRV1\(^-\) cells emerging in the washouts (Fig. 5D). LgyLRV1 copy number was previously estimated as 24-100 by competitive PCR assay (48). To assess LRV1 copy number independently in the clonal LgyLRV1\(^+\) line studied here, we isolated total RNA quantitatively from a known number of cells, and estimated LRV1 copy number by quantitative reverse-transcriptase PCR, using a standard curve established from a cloned LRV1 genome (Methods). This yielded an estimated average LRV1 copy number of 15 ± 0.9 / cell (n=3), consistent with range estimated from the rate of drug induced loss above.
Secondly, after ‘washout’ 2CMA treated parasites, which originally showed homogeneous low levels of LRV1, now reverted to biphasic populations showing WT or ‘negative’ LRV1 levels. The recovery of the WT population suggests that there may be a ‘set point’ for LRV1 levels. As only populations but not clones were studied we cannot be sure that this occurred intracellularly, however the rapidity with which LRV1 levels rebounded suggests this may be more likely.

3.4.8 – Rapid recovery of matched clonal WT and LRV1-cured lines

Our findings suggested that it should be relatively easy to recover LRV1⁻ clonal lines from the 2CMA-treated population. However, we were concerned that despite small effects on growth, the relatively high concentration of 2CMA used above could itself have unwanted selective effects on *Lgy*. Support for this concern arose when in pilot studies, several clonal lines obtained after growth in 100 μM 2CMA lacked LRV1, but showed decreased growth inhibition by 2CMA. Thus we repeated the LRV1 cure using 10 μM 2CMA, a concentration showing less of an effect on parasite growth but retaining strong inhibition of LRV1 levels (Fig. 3). Again, loss of LRV1 proceeded homogeneously (Fig. 6A). When clonal lines were recovered directly by plating from this population, very few were LRV1⁻ (1/30). However, if the population was allowed to grow in the absence of 2CMA for another ~6 cell doublings (washout), a ‘bimodal’ population for LRV1 capsid levels emerged, as seen previously. Analysis of 12 clonal lines obtained by direct plating from this ‘washout’ population showed that 6 exhibited LRV1 capsid levels/profiles identical to the *LgyLRV1⁻* control, while 2 showed profiles identical to the *LgyLRV1⁺* parent (representatives shown in Fig. 6B). Interestingly, 4 lines showed more complex profiles, with populations showing range of intensities spanning those from LRV1⁻ to LRV1⁺ controls (representative shown in Fig. 6B). These complex lines were not studied further.
The ‘set point’ hypothesis predicts that upon further growth, those lines would ultimately revert to bimodal populations.

We chose two LRV1\(^+\) and LRV1-cured lines which had experienced identical 2CMA treatment and culture manipulations. Growth tests confirmed these were not resistant to 2CMA, and RT-PCR and western blot tests confirmed the presence or absence of LRV1 (Fig. 6 C, D). These clones thus constituted ‘matched’ WT and LRV1-cured lines appropriate for subsequent studies of LRV1 effects.

**3.4.9 – LRV1 correlates with increased cytokine secretion and mouse infectivity.**

With matched 2CMA-treated LRV1\(^+\) and LRV1\(^-\) (cured) lines, we asked whether LRV1 was correlated with elevated pathology and hyperinflammatory responses as expected (7, 12). Infections were performed with bone marrow derived macrophages in vitro, followed by assays for secretion of two characteristic LRV1-dependent cytokine reporters, IL6 and TNF\(\alpha\). Cytokine secretion induced by the LRV1\(^+\)/2CMA treated lines was comparable to that of the parental LgyLRV1\(^+\) line, while cytokine secretion induced by the 2CMA cured lines was considerably less, and comparable to that of the LgyLRV1\(^-\) control (Fig. 7A, B).

Infections of susceptible BALB/c mice were performed followed by measurement of pathology (footpad swelling) and bioluminescent imaging of parasite numbers. A strong LRV1-dependency for both pathology and parasite abundance was observed in comparisons of the ‘matched’ 2CMA-treated LRV1\(^+\) vs. LRV1\(^-\) (cured) lines (Figs 7C, D). Importantly, the response to the 2CMA-treated LRV1\(^+\) lines closely matches that to the control parental LgyLRV1\(^+\) line and similarly, the response to the 2CMA-treated LRV1\(^-\) negative line closely matches that to the LgyLRV1\(^-\) control (Figs 7C, D), both of which were studied previously (7).
3.5 – Discussion

In this study we report the first compounds specifically targeting the LRV1 dsRNA virus from *L. guyanensis* and Totiviruses in general. Our findings have relevance for the specific therapeutic inhibition of *Leishmaniavirus*, basic studies of viruses within the Totiviridae, the development of anti-virals directed against dsRNA viruses generally, and the development of new tools for assessing the role of LRV1 in elevating *Leishmania* pathogenicity.

To facilitate the search for LRV1 inhibitors, we first developed a capsid flow cytometry assay to rapidly monitor LRV1 capsid levels (Fig. 1). This assay can be performed in only a few hours, and while these studies employed it in a relatively low throughput manner, it should be scalable for higher throughput. The results were confirmed by anti-capsid or anti-dsRNA western or slot blotting, or quantitative RT-PCR (Figs. 6C, D). Additionally, this assay provides useful information about the cellular heterogeneity of LRV1 levels not readily achievable by other methods, which informed studies probing the inheritance of LRV1 as well as in the generation of LRV1-negative lines.

We focused on known antivirals for several reasons: first, despite significant advances in targeting many retroviruses, DNA viruses or single-stranded RNA viruses, very little effort or progress has been devoted on inhibition of dsRNA viruses. Thus there seemed a reasonable potential for ‘repurposing’ known antivirals against the dsRNA *Leishmaniavirus*. Moreover, since many antivirals are nucleoside analogs and that *Leishmania* is a purine auxotroph (31) the pharmacokinetics of drug uptake and metabolism could well favor the efficacy of such compounds against *Leishmaniavirus*. As a collateral benefit, these studies had the potential to uncover new lead inhibitors against *Leishmania* itself, as auxotrophy has prompted many investigators to target purine metabolism for anti-leishmanial therapy. Several new compounds
not previously reported to inhibit *Leishmania* were identified (Fig 2; Tables S1,S2), but were not pursued further here.

We identified two compounds which showed preferential inhibition of LRV1, 2’-C-methyladenosine or 7-deaza-2’-C-methyladenosine (Figs. 3, S1). The two active compounds were effective in the micromolar range, with >20-fold selectivity for LRV1 versus L. guyanensis growth inhibition and were also active against *Lbr*LRV1, albeit with somewhat less selectivity over growth. The EC$_{50}$ measured using dsRNA or capsid levels were similar, with that of the dsRNA being somewhat less, consistent with the anticipated mode of action targeting the RDRP and genome replication. Both compounds have demonstrated activity against Hepatitis C Virus (HCV), where they target the viral RDRP by chain termination (37, 38, 49). By molecular modeling of the *Lgy* LRV1 RDRP domain against other RDRPs such as HCV, we were able to generate a view of the active site including residues putatively binding to the nucleotide substrates (Fig. S4). Notably, these included sites homologous to those mutated in HCV nucleoside analog-resistant lines (50). This supports our working hypothesis that both anti-LRV1 compounds are activated to triphosphates where they act to inhibit RDRP activity. These compounds represent the first such identified against any member of the Totiviridae, and indeed one of the few candidates described inhibiting dsRNA viruses generally.

Common features of the two selective anti-LRV1 compounds include the 2’-C methyl and the adenine base moieties, although 2’C-methyl G and C were inactive against both *Leishmania* and LRV1. A similar pattern was observed for dengue virus RDRP inhibitors, where only adenosine analogs demonstrated anti-viral activity (51). Following uptake, in *Leishmania* most purine nucleosides are metabolized to nucleobases, the major exception being adenosine which is phosphorylated directly by adenosine kinase (31). This could contribute to the
superiority of 2’-C-methyladenosine analogs. However, all other 2’C-modified analogs tested failed to inhibit LRV1 or Leishmania, including ones bearing adenine or related moieties as the nucleobase (Fig. 2, Tables S1,S2). Other factors may include differential ability to be phosphorylated, often the rate limiting step for antiviral nucleoside activation (52, 53), or susceptibility to nucleoside hydrolases or phosphorylases, which Leishmania possess in abundance (31), and affinity of the phosphorylated analog with the LRV1 RDRP itself. Additional studies will be required to assess the contributions of each of these factors to anti-LRV1 activity and the design of more potent inhibitors.

3.5.1 – Anti-LRV1 agents as a tool for studying Leishmaniavirus replication and biology.

The LRV1 selectivity of 2CMA and 7d2CMA provided the foundation for several studies probing LRV1 biology. Under 2CMA inhibition, a first order kinetic loss of LRV1 was observed, (measured by either capsid or dsRNA genome levels), with a homogeneous 50% loss at every cell doubling (Fig. 5A, B). This fits exactly the prediction assumed by a random inheritance model of LRV1 particles during mitosis. Although widely assumed for the inheritance of most persistent dsRNA viral infections, these findings now provide direct evidence of random segregation. These data also provide a mechanistic explanation for the failure to identify compounds inhibiting both LRV1 and Lgy in our screen, as without continued parasite growth LRV1 cannot be lost by dilution, and indeed may increase somewhat (Fig. 2).

Ultimately LRV1 levels declined to levels approaching those of LRV-free parasites within 3-6 cell doublings following 2CMA treatment (Fig 5). This implies the viral copy number was relatively low, less than 8-64 (2^{3-6}), significantly less than previous estimates of 120 for LgyLRV1 and often many thousands for other Totiviridae (34, 48). However, quantitative analysis of cellular LRV1 and total RNA led to an estimate of about 15, consistent with estimates
of LRV1 abundance from recent whole genome RNA sequencing by our group, to be reported elsewhere. If this unexpectedly low value for LRV1 copy number applies generally to LRV1s in other *Leishmania* strains or species, it could provide a new perspective on the observation that thus far, no images of LRV1 in situ by electron microscopy appear in the literature.

The rapid decline of LRV1 following 2CMA treatment suggested that it would be relatively easy to recover LRV1-free clonal lines. Following washout of 2CMA after 3-6 cell doublings and a brief period of growth without drug, cultures manifested two distinct parasite populations by capsid flow cytometry: one similar to *L. gy*LRV1+ and a second similar to *L. gy*LRV1− (Fig. 5,6). The fraction of *L. gy*LRV1− parasites grew progressively with increasing 2CMA treatment, reaching levels approaching 100%. To recover parasites suited for studies focusing on the biological properties of LRV1-negative parasites, we adopted a protocol in which parasites were treated for only a brief period of time with 10 μM 2CMA, a concentration showing little effect on parasite growth but relatively high inhibition of LRV1 (Fig. 3), followed by brief passaging and then plating on drug-free media. Importantly, this procedure allowed the recovery of both LRV1+ and LRV1− ‘matched’ clonal lines, which had experienced identical treatment, thereby facilitating comparisons probing LRV1 effects (below). Interestingly, in all of these studies the LRV1 levels in ‘washout’ lines showed a strong tendency to recover from the low levels seen in drug to those comparable to LRV1+ controls (Fig. 5). These findings suggest that the LRV1 copy number is maintained at a specific ‘set point’, perhaps through a balance between replication and the RNAi pathway (12, 54). Previous studies examining LRV1 transcripts during growth phase also concluded that LRV1 copy number is regulated (48).

For other fungal dsRNA viruses, treatments engendering cell stress or growth inhibition have been used to generate virus-free lines at significant frequencies, one common example
being the use of CHX to cure the yeast L-A virus (44). Although in one prior study LRV1 cure was obtained during a series of transfection and hygromycin selection steps, this appears to have been successful only once, and our laboratories have been unable to repeat this (12, 46). Here we were unable to show any correlation between LRV1 loss and drug induced stress or growth inhibition with CHX, the ergosterol synthesis inhibitor clotrimazole, or within the large panel of test compounds (Figs. 2,4, S2; Table S1,2). Thus LRV1 appears to be relatively stable to growth inhibitory stresses. However, given its relatively low cellular copy number (<20), on a strictly probabilistic basis LRV1-negative variants might occur at a low frequency, which occasionally may emerge or be recovered by methods more sensitive than employed here.

3.5.2 – Antiviral cures and the generation of isogenic LRV1⁻ lines for the study of LRV1⁻-dependent virulence.

2CMA treatment enables the controlled and reproducible generation of matched LRV1⁺ and LRV1-cured lines without difficulty. In vivo, 2CMA-cured LRV1⁻ parasites showed less pathology and lower parasite numbers and induced less cytokine secretion, than LRV1⁺ parasites, comparable to the single spontaneous LRV1⁻ lines described previously (Fig. 7). Thus our LRV1’ ‘toolkit’ now includes two independent, reproducible and efficient methods for generating isogenic LRV1 negative lines, which will facilitate tests probing the biology of LRV1-dependent pathogenicity in diverse parasite backgrounds. Depending on the relative selectivity of the antivirals and the presence of an RNAi pathway, one method may be superior for a given Leishmania species or strain.
3.5.3 – The potential for anti-totiviral therapy in the treatment of dsRNA-bearing parasites and disease.

There are now ample data suggesting that LRV1 contributes to the severity in human leishmaniasis (6, 13, 17, 19, 20, 55), suggesting that anti-LRV1 inhibitors could be clinically useful, alone or in conjunction with existing anti-leishmanials. Unfortunately, pharmacokinetic studies of the two compounds studied here in mammals suggest that neither of these are good candidates for testing of this hypothesis just yet, as the concentration needed for LRV1 elimination (10 μM) is above the maximum achievable serum concentration in various mammalian models, typically less than 1 μM (38, 49, 56). Thus, future efforts must focus on the development of compounds with higher potency targeting LRV1, without significant human host toxicity. For therapeutic purposes a compound simultaneously targeting both would likely be superior. However, because Leishmania growth is required for LRV1 to be lost by progressive dilution (Fig. 5), a screening method different than that employed here will be required to detect such agents. Dilutional loss following anti-LRV1 inhibitor treatment in vitro predicts that very low levels of LRV1 could persist after treatment in vivo, whether measured on a total or per cell basis (Fig. 5). Importantly, previous data show that below a certain threshold parasites bearing low LRV1 levels are controlled as effectively as LRV1-negative lines (7).

Our studies also raise the possibility of treating other diseases caused by protozoans bearing dsRNA viruses which show endogenous virus dependent pathogenicity, including Totiviridae present within Trichomonas vaginalis (Trichomonasvirus), Giardia lamblia (Giardiavirus), or Eimeria (Eimeravirus) (34, 57) and Partitiviridae within Cryptosporidium parvum (Cryspovirus) (58, 59). Potentially, agents targeting these putative pathogenicity factor viruses could prove similarly valuable for laboratory studies of these viruses as well.
3.6 – Acknowledgements

This work was supported by NIH grants RO1AI029646 and R56AI099364 (SMB), grants FNRS N° 3100A0-116665/1 and IZRJ3_164176/1 (NF), Sigma-Aldrich Predoctoral and the Sondra Schlesinger Graduate Student Fellowships (JIR), and the Division of Infectious Diseases (FMK). We thank N.S. Akopyants, E.A. Brettmann, D.E. Dobson, L-F. Lye and S. Schlesinger for discussions and comments on this manuscript, Chantal Desponds, Florence Prevel and Haroun Zangger for excellent technical assistance, and Jean Patterson (Texas Biomedical Research Institute, San Antonio) for providing capsid antisera.

3.7 – References


38. Carroll SS, et al. (2003) Inhibition of hepatitis C virus RNA replication by 2'-


3.8 – Tables

Table 3-S1: Compounds studied

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92
### Other nucleoside/nucleotides

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### Nucleobase analogs

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### Cysteine Proteinase Inhibitors

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Abbreviations: DNAV, DNA viruses; RNAV, RNA viruses; RTV, retroviruses.

References for Table S1:


2. Olsen DB, et al. (2004) A 7-deaza-adenosine analog is a potent and selective inhibitor of


Table 3-S2: Parasite growth and LRV1 levels in response to test compounds

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Compound names and abbreviations (Table S1) are provided in column 1. Inhibitors were dissolved and used as indicated in column 2. Parasite density was measured after 2 d in culture and normalized to the percent growth of the diluent-treated control in column 3. LRV1 capsid signal was assessed at the same time as growth in column 4. Columns 5 and 6 provide the rank position for each compound as depicted in Fig. S2. Red shading represents inhibition of L. guyanensis. Green shading represents inhibition of LRV1. Yellow shading denotes control parameters. N/A, not applicable.

*Actual rank: 1 = 42, 2 = 12.
Figure 3-1: Anti-LRV1 capsid flow cytometry.

LgyLRV1\(^+\) and LgyLRV1\(^-\) parasites were fixed and permeabilized followed by staining with increasing dilutions of anti-capsid antibody and fluoresceinated secondary antibody. A) Profiles obtained with LgyLRV1\(^+\) (solid line) and LgyLRV1\(^-\) (filled) after selection for single cells. A representative experiment is shown, performed at a dilution of 1:16,000; subsequent studies were performed using a dilution of 1:20,000 (n>11). B) Mean fluorescence of
$L_{gy} LRV1^{+}$ (■) and $L_{gy} LRV1^{-}$ (□) for each antibody dilution. The ratio of $LRV1^{+}/LRV1^{-}$ staining (●) is plotted as a solid line.
Figure 3-2: Antiviral inhibition of Lgy growth vs. LRV1 inhibition.

The figure shows data from Table S2 plotted; LRV1 capsid levels (Y-axis) vs. Lgy growth (X-axis). The large dashed gray circle marks compounds (black dots) showing little effect on LRV1 or Lgy, the red circle marks compounds preferentially inhibiting Lgy growth, and the green circle marks compounds preferentially inhibiting LRV1, blue dots depict 2°C substituted nucleosides without anti-LRV1 activity. LgyLRV1+ and LgyLRV1− controls are shown in brown. Abbreviations for compounds discussed further in the text can be found in Table S1.
Figure 3-3: 2CMA and 7d2CMA inhibition of Lgy growth and LRV1 capsid or RNA levels.

The figure shows the rate of growth or LRV1 capsid levels (y-axis) as a function of drug concentration. A), 2CMA; B), 7d2CMA. Growth rate (●, solid line) and normalized LRV1 capsid (□, dashed line) or RNA (Δ, dashed line) are shown. The results of one representative experiment are shown for 2CMA (n = 2 for RNA and capsid) and a single experiment for 7d2CMA.
**Figure 3-4: LRV1 levels are unaffected by agents inhibiting Lgy growth.**

A) *LgyLRV1* was treated with 10 nM cycloheximide (CHX; □, dashed line), 100 nM CHX (○, dashed line), or no treatment (■, solid line). After 72 hours, cells treated with 100 nM CHX were placed into fresh media (●, dashed line). B) Profiles obtained by LRV1 flow cytometry after 48 h growth for WT (shaded) or cells treated with 100 μM CHX (solid line), or 10 μM CHX (dashed line). C) Plot of growth rate of *LgyLRV1* (●) or LRV1 capsid levels (□, dashed line) after 48h propagation in increasing concentrations of CHX. D) As in panel C but for clotrimazole (CTZ). A representative experiment is shown (n = 3).
Figure 3-5: Kinetics of and cellular distribution of LRV1 loss after treatment with 100 μM 2CMA.

A and B) LgyLRV1⁺ was inoculated into media without (●) or with (□,Δ) 100 μM 2CMA, and growth and LRV1 capsid (□, dashed line) and RNA levels (Δ, dashed line) measured by capsid flow cytometry (A) or qRT-PCR (B). For A, results at each time are shown normalized to LRV1⁺ and LRV1⁻ control levels using the formula log₂ ((2CMA Treated - LRV1⁻)/( LRV1⁺ - LRV1⁻)). For B, the log₂ ddCT values are shown. A theoretical 1:2 dilutional loss is shown (thin gray line); error bars represent ± 1 SD. C) LRV1 capsid flow cytometry of control parasites (top panel) and populations grown for 1, 3, 4 or 6 cell doublings in 100 μM 2CMA. D) LRV1 capsid flow cytometry of parasites grown for 3, 4 or 6 doublings in 100 μM 2CMA, and then grown for an addition 6 cell doublings in drug-free media (washouts). Thick and thin gray dashed lines represent LgyLRV1⁺ and LgyLRV1⁻ respectively.
Figure 3-6: Generation of matched LRV1+ and cured lines after limited 10 μM 2CMA treatment.

A). Workflow for treatment of parasites with 10 μM 2CMA prior to isolation of clonal lines. First drug treatment for 6.4 cell doublings generates a population containing low average LRV1 levels, then the washout for 6 cell doublings allows resolution into fully negative or LRV1+ lines. B). Representative LRV1 capsid profiles for a cured line (Lgy clone 10-5), a WT-like line (Lgy clone 10-10) and a mixed profile line (Lgy clone 10-1; for clarity the leading ‘10’ is omitted from the figures). C). RT-PCR tests confirming presence or absence of LRV1 in treated lines. RT+, reverse transcription performed prior to PCR; RT-, no reverse transcription step. M, 1 kb+ ladder, Invitrogen. The expected LRV1 capsid and β-tubulin amplicons of 496 and ~450 nt were found. D). Western blotting with anti-Lgy LRV1 capsid antisera confirms absence of LRV1 in cured lines Lgy 10-5 and 10-6. M, molecular weight marker. The arrowhead marks the position of the 95 kDa LRV1 capsid band.
Figure 3-7: Matched 2CMA-treated LRV1+ and LRV1- cured lines recapitulate LRV1-dependent virulence.

A, B). Cytokine secretion by BMM infected 24 hr after infection with Lgy lines or treatment with poly I:C (2 μg/ml), M = media. A), TNFα; B), IL-6. The figure shown is representative of three experiments each done in triplicate; error bars represent ± SD. C, D):

Infections of matched 10 μM 2CMA treated LgyLRV1+ and LgyLRV1-. Parasite numbers (luminescence from luciferase reporter) (C) or footpad swelling (D) was measured at the peak of the infection (28 days). Each bar represents pooled data from 8 mice total, 4 for each Lgy line used. LRV1+ (clones 10-9,10-10) and LRV1- (clones 10-5,10-6) lines are shown; error bars represent ± SD. Data for control parasites are replotted from Ives et al. (7).
Figure 3-S1: Structures of compounds showing activity against LRV1 relative to adenosine.
Figure 3-S2: Inhibition results ordered by effects on relative LRV1 (A) or Leishmania guyanensis growth (B).

Dashed lines show the WT control growth rate (red) or $L_gyLRV1^+$ or $L_gyLRV1^-$ capsid levels (blue).
Figure 3-S3: LRV1 inhibition by 2CMA is insensitive to exogenous adenine and does not show synergy with allopurinol.

(A) Plot of growth rate of LgyLRV1⁺ (●) or LRV1 capsid levels (○) after 48-h propagation in increasing concentrations of allopurinol. LRV1 percentages were calculated relative to untreated controls. (B) As in A, but with APP. (C) Effect of increasing concentrations of adenine on LgyLRV1⁺ treated with 100 μM 2CMA for six cell doublings (○, dashed line) or without 2CMA (●, solid line). LgyLRV1⁻ (●) is shown for a reference without adenine. (D) The EC₅₀ for 2CMA inhibition of LRV1 after 48 h is unaltered in the presence of allopurinol. The geometric mean capsid intensity is plotted relative to an untreated control. None (●, solid line), 0.1 μM (■, solid line), 1 μM (●, dashed line), 10 μM (■, dashed line), and 100 μM (○, dashed line). Results from a single experiment are shown, other than C (n = 3).
Figure 3-S4: Active site model for L. guyanensis RDRP.

A) Overall structural alignment of the Lgy LRV1 RDRP core domain’s predicted structure (green) to a crystal structure of the Hepatitis C virus RDRP (light blue; PDB 4wti; (1)) created with the UCSF Chimera MatchMaker tool (2). For clarity, only the portion of the HCV RDRP (residues 103-422) that corresponds to the LRV1 RDRP core is shown. The HCV RDRP structure contained bound RNA and GDP. The GDP is shown in this figure to locate the NTP binding pocket. The Lgy LRV1 RDRP structure was predicted using the intensive method on the PHYRE2 web service (3), which yielded a high-confidence (≥90%) region between residues 337 and 660. Given just this core region, PHYRE2 produced a very high-confidence structure (100% confidence over 94% of residues) with an active site very similar to the HCV structure. B) Predicted structure of the nucleotide binding pocket in the LRV1 RDRP. The GDP molecule...
from panel A is shown for clarity. Surface colored yellow represents the locations of residues forming a binding site predicted by the 3DLigandSite server with high confidence (average MAMMOTH score 29.7, where ≥7 is significant) (4). Areas colored green mark residues which, when mutated in the HCV RDRP, confer resistance to the 2’-C-methyl family of nucleoside analogs (5-7). The “Rotamers” tool in UCSF Chimera was used to fix side-chains given unfavorable conformations by the PHYRE2 server (8). C) Table of predicted binding site residues in LgvM4147 LRV1 RDRP and their corresponding residues in the HCV RDRP.

References for Figure 3-11.


Chapter 4: Accumulation of 2’C-methyladenosine triphosphate in *Leishmania guyanensis* enables specific inhibition of the *Leishmania* RNA virus 1 polymerase
Preface

SMB and JIR designed the experiments. JIR performed the experiments and analyzed the data with technical assistance from George Lye and Erin Brettmann. The simulations were designed with input from Dr. Eric Galburt. The first draft of this chapter was written by JIR and the final version presented here incorporates comments from SMB.
Abstract

*Leishmania* is a widespread Trypanosomatid protozoan parasite causing significant morbidity and mortality in humans. The dsRNA virus *Leishmania* RNA virus 1 (LRV1) chronically infects some *Leishmania* strains, where it leads to increased parasite numbers and pathology in murine leishmaniasis models, and correlates with increased treatment failure and relapse in human disease. Previously, we reported that 2’-C-methyladenosine (2CMA) was a potent inhibitor of LRV1 in *L. guyanensis* (*Lgy*) and *L. braziliensis*, rapidly eradicating LRV1 at concentrations around 10 µM. Here, we probe the mechanism of 2CMA inhibition. Inhibition of RDRP activity by 2CMA triphosphate (2CMA-TP) was assayed by measuring incorporation of \([\alpha^{32}\text{P}]-\text{UTP}\) by cesium chloride gradient-purified *Lgy* virions. IC50s ranged from 130 to >600 µM depending on the density of the virion particle (empty, ssRNA- and dsRNA-containing) and product formed. Inhibition was specific and was not seen with 2CMA or dATP. *Lgy* parasites incubated *in vitro* with 10 µM 2CMA accumulated 2CMA-TP to 410 µM, well beyond that needed to inhibit LRV1 replication. We developed a quantitative model that showed good agreement between the degree of LRV1 RDRP inhibition and LRV1 levels, supporting a model where 2CMA exerts its effects through metabolism to 2CMA-TP and accumulation to levels sufficient for RDRP inhibition and LRV1 loss. This attests to the powerful *Leishmania* purine uptake and metabolism pathways that allow even a weak RDRP inhibitor to effectively eradicate LRV1 at micromolar concentrations. These data suggest the possibility that 2’C-methyl-substituted adenosine analogs may have potential therapeutic applications to ameliorate the increased pathogenicity conferred by LRV1.
4.2 – Introduction

The neglected tropical disease leishmaniasis is caused by various species of the genus *Leishmania*, which are single-celled eukaryotic parasites transmitted by multiple species of sand flies. (1) In South America, infection by *Leishmania guyanensis* (*Lgy*) or *Leishmania braziliensis* (*Lbr*) initially causes a self-resolving skin lesion (cutaneous leishmaniasis, CL). In some cases (primarily *Lbr*), however, the infection re-emerges and parasites metastasize to other locations, especially the mucus membranes (mucocutaneous leishmaniasis, MCL) (2). The factors determining disease progression and responsiveness to treatment are unclear, but are thought to be both host- and pathogen-derived. (3,4)

Many isolates of *Leishmania* within the subgenus Viannia, primarily *Lbr* and *Lgy*, bear a single-segmented dsRNA virus known as *Leishmania* virus 1 (LRV1) (5-7). Previous work has shown definitively that mice infected with parasites containing the endobiont LRV1 exhibit greater pathology, higher parasite numbers, and increased metastasis (8,9) These studies have benefited from the availability of isogenic LRV1+ or LRV1- lines, generated spontaneously or by defined methods such as RNA interference or antiviral drug treatment (10-12). The role of LRV1 in human leishmaniasis has been more challenging to establish definitively. When comparing rates of CL and MCL, some studies find that LRV1+ strains generate more MCL (13-15), while others do not (16,17). These discrepant findings may be explained by other parasite or host factors known to play a significant role in the development of MCL (18-20). Alternatively, co-infections with viruses, especially those that can induce the Type I interferon responses, were shown recently to exacerbate *Lgy* pathology and metastasis (21,22). The presence of LRV1 in

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1 The abbreviations used are: *Leishmania guyanensis* (*Lgy*), *Leishmania braziliensis* (*Lbr*), *Leishmania* RNA virus 1 (LRV1), 2'-C-methyladenosine (2CMA), 2'-C-methyladenosine triphosphate (2CMA-TP), RNA-dependent RNA polymerase (RDRP), 7-deaza-2'-C-methyladenosine (7d2CMA), cesium chloride (CsCl), low-density (LD), medium density (MD), high density (HD).
clinical isolates of *Lbr* or *Lgy* correlates with drug-treatment failure (14,16), which could be explained by the increased parasite numbers or altered host responses predicted from animal models (8,18,23). Treatment failure and relapse rates are also significantly higher in infections where *Lbr* or *Lgy* bear LRV1 (14,16). Overall, there is good reason to postulate a role for LRV1 in increasing disease severity in human leishmaniasis (18).

Like most other Totivirus species, LRV1 is neither shed nor infectious and is inherited vertically (6,24). Indeed, phylogenetic evidence suggests that LRV1 strains may persist and co-evolve with their *Leishmania* host over millions of years (6). LRV1 follows a typical Totivirus life cycle (Fig. 1) (25), where mature virions contain one dsRNA genome and several RNA-dependent RNA polymerases (RDRPs). The viral RDRP transcribes positive-sense genomic ssRNAs encoding two large overlapping reading frames encoding the capsid and RDRP, respectively. The second is thought to be translated via a frameshift, generating a capsid-RDRP fusion (26-28). The capsid monomers then self-assemble into immature virions (29), incorporating the positive-sense ssRNA transcript, which the RDRP replicates into the mature dsRNA genome.

Importantly, murine vaccination using the LRV1 capsid results in significant protection against LRV1+ *Lgy* (30), suggesting that therapies targeting LRV1 specifically might aid in reducing disease pathology. One such approach is specific inhibition of LRV1 by small molecules. Previously, we surveyed a small library of antiviral nucleosides and identified two closely-related adenosine analogs, 2′C-methyl adenosine (2CMA) and 7-deaza-2′-C-methyl adenosine (7d2CMA), which specifically inhibit LRV1 replication in *Leishmania* cells (Fig. S1). These compounds exhibited EC50s of 3-5 µM for viral inhibition and rapidly eradicate LRV1 at concentrations above 10 µM. This allowed us to easily create isogenic LRV1- lines (11). These
studies did not address the mechanism of anti-LRV1 activity, which was postulated to arise through direct inhibition of the LRV1 RDRP by the triphosphorylated form of 2CMA. Here we provide support for these hypotheses, as well as evidence that hyper-accumulation and retention of 2CMA-TP is sufficient to overcome its relatively weak inhibition of the LRV1 RDRP activity.

4.3 – Results

4.3.1 – Purification and separation of virion populations.

RDRP assays were carried out with LRV1 virions purified from Lgy strain M4147 by an established cesium chloride (CsCl) equilibrium ultracentrifugation procedure (5,31). Following separation, virus particles were detected and quantified by their reactivity with an anti-capsid antibody (Fig. 2). We reproducibly observed three overlapping ‘peaks’, designated low-, medium-, and high-density (LD, MD and HD) in order of increasing density. In previous studies of the yeast L-A Totivirus, similar peaks were shown to correspond to virions that were primarily ‘empty’ or contained ssRNA or dsRNA, respectively (32,33). The densities of the Lgy LRV1 LD, MD and HD peaks were 1.29, 1.36 and 1.41 g/mL, in good agreement with the densities of L-A virus particles bearing ssRNA- and dsRNA- (1.31 and 1.41 g/mL, respectively) (34). Preliminary data from S1 nuclease digestion of viral RNA from these fractions were consistent with these assignments (data not shown).

4.3.2 – In vitro assay of LRV1 RDRP activity.

To measure RDRP activity, purified virions were allowed to incorporate [α-32P]UTP in the presence of the remaining nucleoside triphosphates for 1 hr, a time chosen to allow one round of transcription or replication of the viral genome (31). RNA was purified and separated by native gel electrophoresis, and the products were visualized and quantified. Two products were found: one about 5 kb, presumably corresponding to the full-length LRV1 genome, and a
smaller, heterogeneous product ranging from 0.1 – 0.5 kb, which we attributed to abortive transcripts (Fig. 3A). Neither extending the incubation time nor increasing the concentration of UTP significantly altered the profile obtained (not shown). Importantly, neither full-length nor small products were produced by corresponding preparations from LRV1-negative parasites (Fig. 3B), and thus were specific to LRV1.

4.3.3 – 2CMA-TP specifically inhibits viral RDRP activity.

Incubation of the three LRV1 populations with 2CMA-TP reduced synthesis of both the full-length and small RDRP products (Figs. 3 - 4). The synthesis of each product was quantitated and normalized to that obtained with drug-free controls, from which IC50s were calculated (Table 1). These data showed a range of IC50s, from 130 μM for full-length product synthesis by LD virions to over 500 μM for the small products (Table 1, Fig. 4). These IC50s were unexpectedly high, greatly exceeding the extracellular concentration of 2CMA shown previously to cause 50% inhibition of LRV1 abundance (~3 μM) (11). We were concerned that this arose artificially from 2CMA-TP degradation during the assay. However, HPLC tests of RDRP reactions before or after the 1 hr incubation showed that only 3.7 ± 0.6% (n = 3) of the 2CMA-TP was degraded to what appeared to be 2CMA-DP during the course of the assay. Thus, drug degradation was unlikely to significantly reduce its potency.

As anticipated, 2CMA did not measurably inhibit RDRP activity when tested at concentrations up to 1000 μM (Fig. 5). Similarly, dATP, which lacks both the 2’-hydroxyl and methyl groups of 2CMA (Fig. S1), failed to inhibit RDRP activity at the highest concentration tested (600 μM; Fig. 5). These data suggest that despite its relatively low potency, 2CMA-TP inhibition of LRV1 RDRP activity was specific under these conditions.
4.3.4 – 2CMA activation to 2CMA-TP and accumulation in parasites.

To account for the relative sensitivity of Lgy LRV1 to 2CMA compared to the insensitivity of LRV1 RDRP activity to inhibition by 2CMA-TP, we hypothesized that parasites take up and activate 2CMA, accumulating high 2CMA-TP concentrations. We first identified an HPLC protocol capable of resolving both synthetic 2CMA-TP and a smaller peak we presume to be 2CMA-DP from natural ribonucleotides and the internal standard, dGTP (Fig. 6A-B).

Standard mixtures of known concentration were used to generate a standard curve relating peak area to 2CMA-TP amounts (Fig S3). We then tested several protocols for extracting parasite nucleotides and determined that extraction with 1:1 acetonitrile:water performed best (Methods). Using this protocol, we compared the nucleotide profiles of LRV1+ Lgy grown in the presence or absence of 10 µM extracellular 2CMA for 20 hours, a time chosen because it corresponds to about two rounds of parasite replication. Under these conditions, we observed a peak co-eluting with synthetic 2CMA-TP that was absent from untreated parasites (Fig. 6B), establishing the parasite’s capacity to phosphorylate 2CMA.

We then measured steady-state levels of intracellular 2CMA-TP following incubation of late-log-phase parasites with 2CMA for 20 hours. To determine the intracellular concentration of 2CMA-TP under these conditions, we measured the average volume of the parasites (roughly 23 fL; see Methods). The steady state concentration of 2CMA-TP was measured at external 2CMA concentrations of 1 - 10 µM, bracketing the EC50 for LRV1 inhibition (3 µM) and extending to a concentration sufficient to completely inhibit LRV1 replication (10 µM) (11). Over this range of concentrations, internal 2CMA-TP levels exceeded external 2CMA concentrations by 40- to 80-fold (Fig. 7A), attesting to the potency of the parasite’s purine salvage pathway.
When propagated in 10 µM external 2CMA, the intracellular 2CMA-TP concentration reached 410 ± 110 µM (n=4) (Fig. 7A). This is well in excess of the minimum IC50 seen for RDRP inhibition (130 µM for full-length products from LD virions; Table 1). When treated with 3 µM external 2CMA, which is the EC50 for LRV1 inhibition, the intracellular 2CMA-TP concentration was 152 ± 43 µM (n = 4), comparable to the RDRP IC50. Finally, at 1 µM external 2CMA, a concentration with minimal effect on LRV1 levels, the intracellular 2CMA-TP concentration was 78 ± 9.0 µM, well below the lowest IC50 for RDRP inhibition (n=4) (Fig. 7A). Thus, the steady-state levels of 2CMA-TP corresponded reasonably well to the effects on LRV1 inhibition predicted from RDRP inhibition alone.

Finally, we measured the rate of 2CMA-TP accumulation by adding 10 µM 2CMA to late-log-phase cells and measuring the intracellular 2CMA-TP concentration after 0.5, 1, 2, 4, or 8 hours (n=2). We observed measurable 2CMA-TP accumulation as early as 1 hour after 2CMA addition (Fig. 7B). The concentration surpassed the lowest IC50 for RDRP inhibition (130 µM) within 4 hours and reached the 20-hour steady-state concentration by 8 hours. This indicates that intracellular 2CMA-TP reaches inhibitory concentrations relatively quickly after exposure to 2CMA.

4.3.5 – Parasites retain 2CMA-TP after removal of drug pressure.

It has been shown that some nucleoside analogs, once phosphorylated by cells, are retained as nucleoside analog-triphosphates for a significant period following removal of cells from the nucleoside itself (35). Because longer retention may increase drug efficacy, we measured parasite retention of 2CMA-TP. Lgy M4147 LRV1+ parasites were incubated for 18 hr in 10 µM 2CMA, washed, and resuspended in drug-free medium. Remarkably, parasites retained approximately 50% of their accumulated 2CMA-TP after 4 hours, while after 8 hours the
average 2CMA-TP concentration was 170 ± 100 µM (n = 5) (Fig. 8). These values were not corrected for dilution of 2CMA-TP due to parasite replication during incubation in drug-free medium. Correcting for population growth, the intrinsic 8 hour concentration was estimated to be 230 ± 130 µM. Compared to the 0.3-hour half life of 2CMA in rat serum (36), these data establish that once formed, 2CMA-TP has much greater intracellular persistence than the 2CMA serum concentration would suggest.

4.3.6 – Simulating virus inhibition replicates experimental results.

The studies above suggest that inhibition of LRV1 RDRP activity alone may be sufficient to explain the elimination of LRV1 infection by 2CMA. As a further test of this hypothesis, we asked whether a simple computational model using the relative rates of parasite and viral replication could quantitatively describe our experimental data. We developed a model based on Gibson and Bruck’s next-reaction modification to Gillespie’s stochastic simulation algorithm (37), which directly simulates the occurrence of individual events over time by picking the next event-time from an associated probability distribution (Methods; Supplemental File 2). For this model, the two events we are simulating are parasite division and virus replication. Thus, the primary parameters defining the model are parasite and virus replication rates as defined by the experiments presented in this study. Replication rates were calculated based on the parasite population doubling time. We used the experimentally-measured parasite doubling time of 7.5 hours and assumed that in the absence of drug pressure the relative parasite and virus replication rates were identical. The effect of 2CMA was modeled by adjusting the ratio of LRV1 to parasite replication rates (V:P) based on experimental values determined in this study (explained in detail below). Simulations began with an initial population of 1000 cells, each infected with 16 LRV1
particles (11). Using these conditions, the simulation correctly maintains the LRV1 population at an average of 16 virions per parasite over time in the absence of drug pressure (Fig. 9A).

To model LRV1 elimination by 2CMA, we decreased the LRV1 replication rate so that the ratio of virus to parasite replication rates (V:P) was 1:2, 1:3, and 1:4. This yielded LRV1 loss profiles which closely matched those determined experimentally (11) (Fig. 9A; solid lines simulation, dashed lines experimental). To see if these replication rate ratios were consistent with our in vitro experimental results, we compared them to the degree of inhibition caused by 2CMA-TP on RDRP activity.

LRV1 RDRP activity on substrates from low density virions was most sensitive to 2CMA-TP in vitro (IC50 130 µM for full-length product synthesis by LD virions; Table 1; Fig. 4A). Therefore, we assumed that the concentration-dependent effect of 2CMA on the LRV1 replication rate directly depends on the effect of 2CMA-TP on the most sensitive RDRP activity. We plotted both normalized RDRP activity and parasite division rates as a function of 2CMA-TP and 2CMA, respectively (Fig. 9B). Fitting the RDRP inhibition data yielded a function representative of RDRP inhibition by 2CMA-TP and, by extension, of LRV1 replication by 2CMA. This allowed us to calculate the ratio of RDRP activity to parasite division rates for each parasite division point (Fig. 9B, blue line). As can be seen in the graph in Figure 9B, the ratio of rates at 10 µM 2CMA, when RDRP activity is strongly inhibited, falls exactly within the 1:2 to 1:4 range that accurately reproduced LRV1 loss in our model. Specifically, at 10 µM extracellular 2CMA, intracellular 2CMA-TP is 410 µM, and the ratio of rates is ~1:3 (Fig. 9). The results of our simulations suggest that, despite the complexity of viral replication, the system behaves as though RDRP activity is rate limiting for viral replication and our in vitro
measurements of 2CMA-TP inhibition are consistent with our in vivo measurements of viral elimination time courses.

4.4 Discussion

Previously, we showed that two 2’-C-methyl-adenosine analogs selectively inhibit the replication of Lgy and Lbr LRV1, to the point that LRV1 could be eradicated with exposure to 10 µM inhibitor (11). In that study the mechanism of action was presumed but not shown to follow the classic antiviral nucleoside paradigm of uptake, conversion to the nucleoside triphosphate, and inhibition of the viral RDRP. In this study, we provide evidence that this is in fact the case for Lgy LRV1 inhibition following 2CMA treatment.

We first established an assay for RDRP from partially-purified virions, following the incorporation of radiolabeled UTP. As virions were purified on CsCl density gradients, we assayed low, medium and high density fractions, which are virion mixtures where the predominant species correspond to different steps of viral maturation (Figs. 1, 2). For all samples, activity was dependent on the presence of LRV1 and yielded two major products, corresponding to the full length viral genome as well as a heterogeneous collection of small and presumably abortive transcripts (Fig. 3). Quantitative analysis showed that overall the IC50s for the full-length product synthesis were lower than measured for small transcript synthesis (130 - 410 vs. 510 – 1000 µM; Table 1), and less for the low density virions than the high density (130/510 vs. 410/1000 µM), although these differences were not quite statistically significant (p < 0.07 or 0.1, respectively; Table 1). These differences may signify different intrinsic sensitivities of the RDRP activity within mature and immature viral particles, perhaps related to the initiation of positive-strand (mRNA) vs negative-strand synthesis. This is the first such report for Totiviruses, for which antiviral drugs have only recently been reported (11). Differential
effects on replicase versus transcriptase have also been seen in reoviruses, where ribavirin triphosphate inhibits replicase but not transcriptase activity (38). Our current studies are limited because the RDRP assay depends on native RNA substrates from incompletely purified virions. This prevents complete separation of RDRP transcription and replication activities, and precludes the use of tightly controlled initiation and elongation assays. More precise work with purified RDRP and well-defined synthetic substrates will be required to fully elucidate the mechanism of action of 2CMA-TP.

2CMA itself was completely inactive for RDRP inhibition, as was dATP (Fig. 5). The lack of 2CMA activity was expected, as this activation to triphosphate form is common and often rate limiting amongst nucleoside analog drugs (39-41). It was shown previously that the triphosphate form of 2CMA, but not the analog itself, was active against the Hepatitis C virus RNA polymerase (42).

Notably, 2CMA-TP inhibition of the LRV1 RDRP activity was not very potent, (>130 µM; Table 1), in contrast to inhibition of LRV1 within parasites by 2CMA exposure (3 µM) (11). We resolved this discrepancy by showing that the parasites avidly scavenged 2CMA from the medium and efficiently convert it to the active triphosphate form (Fig. 6), reaching 2CMA-TP concentrations more than 40-fold above the 2CMA concentration in the medium within 8 hours of drug exposure (Fig. 7). Strong accumulation of toxic anti-leishmanial purines was also noted earlier in studies (43,44). Interestingly, the rate of 2CMA-TP accumulation is similar to the previously-measured accumulation of formycin B triphosphate in L. donovani (43). These results underscore the importance of the purine salvage pathway in designing drugs targeting auxotrophic Leishmania parasites, which must avidly scavenge all naturally occurring purines from their environment (45). In the case of 2CMA, the salvage pathway converts 2CMA-TP, an
admittedly poor inhibitor of the LRV1 RDRP, into a potent tool for eliminating the virus. One particularly important step is likely the adenosine kinase (46,47), which may mediate the initial and often rate limiting phosphorylation of antiviral nucleosides (40,48).

Importantly, the accumulated levels of intracellular 2CMA-TP closely matched the consequences of RDRP and LRV1 loss. At 10 µM external 2CMA, 410 µM internal 2CMA-TP was attained, which was well over the minimal LRV1 RDRP IC50 (130 µM). In contrast, at 1 µM external 2CMA, internal 2CMA-TP concentrations were only 80 µM, well below that needed for RDRP inhibition, and little effect was seen on LRV1 levels (11). These data suggest that inhibition of LRV1 RDRP activity by 2CMA-TP alone is sufficient to account for the rapid loss of LRV1. In further support of this contention, we used a Gillespie (49) simulation to model LRV1 loss, with good correspondence between predictions and experimental data gathered previously (11). While our studies did not examine other potential 2CMA-TP targets, such as the capsid endonuclease (50-54), collectively our data show that the elimination of LRV1 by 2CMA can be largely explained by the direct inhibition of LRV1 RDRP activity by 2CMA-TP.

These and previous studies raise the question of whether treatment of anti-LRV1 agents could be used therapeutically to ameliorate the severity of Lgy and Lbr infections. In animal models, 2CMA has a short serum half life (0.3 hours), although 7d2CMA has a half life of 1.6 hr (55). Interestingly, 7d2CMA shows an EC50 against Zika virus in cultured mammalian cells comparable to those observed against LRV1 (10 µM vs. 5 µM) (56,57). For Zika, regular dosing regimens have been able to achieve sufficient concentrations to show significant inhibition in animal models (56,57), suggesting that it might likewise be possible to achieve inhibition of LRV1 in vivo as well. The rate of 2CMA-TP accumulation also supports the feasibility of LRV1 inhibition, because 2CMA-TP levels reach inhibitory concentrations within 4-8 hours at 10 µM.
2CMA. Rough pharmacokinetic predictions suggest that 7d2CMA levels above 10 µM could be maintained in mice for approximately the same length of time.

Importantly, we also showed that once formed 2CMA-TP is retained for a considerable period of time within parasites, suggesting that even a brief exposure at a sufficient 2CMA dose may lead to prolonged intracellular therapeutic levels of 2CMA-TP (Fig. 8). Assuming that 7d2CMA shows a similar retention profile, as seems likely, the efficacy of both of these compounds may be extended beyond that predicted by serum level.

A second line of inquiry would of course be the development of anti-LRV1 agents with improved potency. Preliminary studies expressing a promiscuous HSV TK gene within *Leishmania* did not increase the spectrum of activity significantly for those analogs tested from our previous study (11), suggesting that the lack of activity may reflect failure to inhibit LRV1 RDRP itself rather than lack of metabolism. Indeed, we found that while high levels of 2CMA-TP were formed in *L. major* strain 5-ASKH bearing the related virus LRV2, no inhibition of virus levels was seen (data not shown). Similarly, we found that several immucillins shown previously to inhibit *Leishmania* nucleoside hydrolases had little effect on drug potencies (Immucillin A, DADMe-Immucillin A, Immucillin H, and DADMe-Immucillin H; data not shown). (58,59) Although nucleoside analogs themselves, they showed no inhibition of LRV1 levels when tested at concentrations up to 100 µM (data not shown). Thus, future efforts focusing on improved potency against the RDRP activity itself may prove most fruitful.

Thus, our work now sets the stage for future studies exploring the possibility that LRV1-targeted therapies may ameliorate the pathology of those *Leishmania* species and strains that bear this fascinating virus.
4.5 – Methods

4.5.1 – Parasite strains and media.

Luciferase-expressing isogenic clones of *L. guyanensis* strain M4147 (MHOM/BR/75/M4147) were utilized for these studies. LRV1+ clone LgyM4147/SSU:IR2SAT-LUC(b)c3 and LRV1− clone LgyM4147/pX63HYG/SSU:IR2SAT-LUC(b)c4 were described previously. (60) For some experiments a LgyM4147/LRV1+ line expressing GFP+ [LgyM4147/SSU:IR2SAT-LUC(b)c3/SSU:IR3HYG-GFP+(b)] was used (provided by E. Brettmann). Schneider’s medium (Sigma Aldrich, St. Louis, MO) was prepared following the manufacturer’s instructions, supplemented with 10% heat-inactivated FBS, 0.76 mM hemin, 2 µg/mL bioppterin, 50 U/mL penicillin, and 50 µg/mL streptomycin, and adjusted to a final pH of 6.5. M199 medium was prepared with 2% heat-inactivated FBS, 2% filter-sterilized human urine, 0.1 mM adenine, 1 µg/mL biotin, 5 µg/mL hemin, 2 µg/mL bioppterin, 50 U/mL penicillin, 50 µg/mL streptomycin, and 40 mM HEPES, pH 7.4 (61). No significant differences were observed in the properties of virus preparations from either medium. Cells were counted using either a hemocytometer or a Coulter counter (Beckton Dickinson).

4.5.2 – Virion fractionation.

Parasites were grown to early stationary phase in M199 or Schneider’s medium (3×10^7 or 9×10^7 cells/mL, respectively). 1×10^10 cells were pelleted at 2200×g for 15 min at 4°C and washed twice with 10 mL ice-cold TMN buffer (100 mM Tris, pH 7.5; 50 mM MgCl_2; 1.5 M NaCl). Cells were then resuspended in 1 mL ice-cold lysis buffer (TMN buffer plus 1 mM DTT, 1× Complete protease inhibitor cocktail (Roche) and 1% (v/v) Triton X-100), homogenized by pipetting 10-12 times with a 1-mL micropipette, and incubated on ice for 20-30 minutes. Lysis was completed by passing the mixture repeatedly through a 27G needle, after which it was
clarified by centrifugation at 15,000 × g for 10 minutes at 4°C. Density gradients were prepared by thoroughly mixing the clarified lysates with enough 10×TMN buffer, saturated CsCl, and distilled water to make 12 mL of solution at a final density of 1.35 g/mL (2.82 M CsCl). Gradients were spun in a pre-chilled SW41Ti rotor (Beckman) at 32,000 rpm and 4°C for approximately 72 hours. Twelve 1-mL fractions were recovered immediately from each gradient using a density gradient fractionator (Isco).

The distribution of capsid protein across each gradient was determined by binding of 50 µL aliquots of each fraction to a nitrocellulose membrane using a Mini-fold II Slot-Blot System (Schleicher & Schuell, Keane, NH). The membrane was incubated on a roller with blocking buffer (2% non-fat powdered milk in PBS) for 1 hour, then stained with 1:2500 rabbit anti-capsid antibody (62) in blocking buffer plus 0.2% TWEEN-20 (TWEEN buffer) for another hour. The membrane was then washed 3 times for 5 minutes in 1×PBS plus 0.1% TWEEN-20 (PBST). Membranes were next incubated in TWEEN buffer for 1 hour with 1:10,000 goat anti-rabbit antibodies conjugated to IRDye 680 (LiCor Biosciences). Finally, the membranes were washed 3× in PBST and once in PBS. Membranes were scanned with an Odyssey Infrared Imaging System (LiCor Biosciences). The density of each fraction was measured by taking its refractive index with an Abbe refractometer (Bausch and Lomb) and converting to density using published formulas (63). Gradient fractions of interest (Fig. 2) were dialyzed twice against 1×TMN and once in 1×TMN plus 20% glycerol (4°C), reaching CsCl concentrations less than 2 µM. Fractions were flash frozen and stored at -80°C prior to use.

4.5.3– RDRP assay.

RDRP activity of purified virions was measured using an [α-32P]UTP incorporation assay described previously(31). Briefly, 20 µL reactions contained 10 mM Tris-HCl (pH 7.5); 150 mM
NaCl; 3 mM MgCl₂; 4 mM DTT; 50 μM each ATP, CTP, and GTP; 20 μCi [α-³²P]UTP; and 10 μL virions. Reactions were incubated at room temperature for 1 hour and quenched by addition of 350 μL TRIzol (Ambion). A corresponding gradient fraction from LRV1-parasites was included as a negative control in each set. RNA was purified using a Direct-Zol RNA miniprep kit (Zymo Research) and run on a native 1.2% agarose-TAE gel in a vertical gel apparatus (Owl Scientific) along with dsDNA sizing standards. The standards lane was excised and stained with ethidium bromide, while the radiolabeled products were detected by exposing an imaging plate for 24 hours and reading it with a FLA-5100 phosphoimager (Fuji). The amount of radiolabeled UTP in each RDRP product was quantified using the gel analysis tool in FIJI/ImageJ (64). Equivalent regions from the negative control reaction were also integrated to calculate the background (Fig. 3).

To study inhibition of the viral RDRP by 2CMA-TP, varying amounts of the compound were added to standard RDRP reaction mixtures. 2CMA-TP was custom synthesized by Jena Bioscience, and its identity was confirmed using electrospray ionization with a Fourier-transform mass spectrometer in negative ion mode (Thermo Scientific). The stock concentration of 2CMA-TP was calculated by UV absorption at 260 nm, assuming that its molar extinction coefficient was identical to ATP. To measure the amount of 2CMA-TP which is non-specifically hydrolyzed over the course of an RDRP reaction, mock reactions were run using LRV1-gradient fractions, cold UTP, and 300 μM 2CMA-TP. The 20-μL reactions were diluted to 80 μL with distilled water and immediately analyzed by HPLC as described below.

4.5.4– Measurement of parasite volumes.

Cultures of WT or GFP-expressing LRV1+ Lgy M4147 were seeded at 2×10⁵ cells/mL and analyzed when they reached early, mid, or late log phase. From each sample, one aliquot was
analyzed by light scattering on a flow-cytometer, while another was immobilized by treatment with 20 mM sodium azide and imaged by spinning-disk confocal microscopy (65). Cell volumes were calculated using a custom ImageJ script (64). (Supplemental Text 1). A standard curve relating forward scattering intensity to measured volume was developed and used to estimate metabolite concentrations (Fig. S2).

4.5.5–Metabolite extraction from Leishmania parasites.

For drug metabolism studies, $10^8$ late-log phase parasites in 5 mL of Schneider’s medium were treated with indicated drug concentrations. Each replicate of each time point was obtained from a separate culture. For accumulation experiments, $10^8$ parasites were harvested after 0.5, 1, 2, 4, and 8 hours in 10 µM drug. To measure steady-state 2CMA-TP levels, $10^8$ cells were grown for 20 hours before harvesting. In ‘washout’ experiments, each culture was grown for 20 hours in 10 µM drug. These cultures were spun down, resuspended in drug-free medium, centrifuged again, and finally suspended in 5 mL drug-free Schneider’s medium. After 2, 4, or 8 hours, nucleotides were extracted from $10^8$ parasites from individual washed cultures and analyzed by HPLC.

For each sample, cells were collected by centrifugation at $2200 \times g$, 4°C for 5 min, resuspended in 1 mL ice-cold PBS and re-centrifuged. The cell pellet was gently re-suspended in 100 µL ice-cold 0.5×PBS plus 7 nmol dGTP as a recovery and elution standard. Although dGTP occurs naturally, its intracellular concentration of approximately 5 µM is well below the limit of detection for this assay and thus does not interfere with its use for this purpose.(66). Cells were immediately lysed by rapidly re-suspending in 900 µL ice-cold 5:4 acetonitrile:water mixture (67 ) and vortexing continuously for 5 min at 4°C. Insoluble debris was pelleted at $16,000 \times g$ for 5 minutes and the clarified extract was transferred to a fresh tube. The solvent was removed by
evaporation in a Savant SpeedVac concentrator (Thermo Scientific) with the heater off and the vacuum pump refrigeration on. Samples were re-suspended with 80 µL distilled water, flash frozen, and stored at -80°C prior to HPLC analysis.

4.5.6– HPLC separation of nucleotides.

Nucleoside di- and tri-phosphates were separated by isocratic HPLC as described (68). Briefly, cel extracts were clarified by centrifuging for 2 min. at 16,000×g and a 20 µL aliquot was injected onto a Zorbax SB-C18 column (5 µm particle size, 250 mm × 4.6 mm, Agilent) and eluted at 1 mL/min with 150 mM KH₂PO₄ (pH 6.0); 4.2 mM tetrabutylammonium hydroxide; and 5.4% methanol. Eluting compounds were monitored by UV absorbance at 254 nm. The elution times of nucleoside triphosphates as well as 2CMA and 2CMA-TP were determined by running them individually. A minor peak present in each standard was presumed to represent the di-phosphate form of that nucleoside. A mixture containing 200 µM ATP, GTP, CTP, UTP, and dGTP was used periodically to assess column performance. Peak areas were integrated using Millenium32 software (Waters), showing that peak area varies linearly with compound amount injected, above 50 pmol (Fig. S3).

4.5.7– Gillespie simulation of LRV1 inhibition.

We modeled the effects of 2CMA treatment on LRV1 using the next-reaction modification to the Gillespie algorithm (code is provided in Supplemental Text 2) (37). The parameters used to define the system were as follows: number of parasites, number of virions per parasite, parasite growth rate, and virus replication rate. All simulations were initialized with 1000 parasites and 16 virions per cell. Each cell and virus was assigned an amount of time remaining until it divided or replicated, respectively. Because these delay times were composed of an unknown but large number of elementary chemical reactions, they were selected from
Gaussian distributions about the mean parasite division and virion replication times, rather than the Poisson distributions used for elementary reactions (37,49). At each step, the event with the shortest time remaining was selected, the simulation time incremented, and the model updated accordingly.

4.6– Acknowledgements

We thank E. Galburt for discussions and advice leading to the Gillespie simulation presented in this work, J. Henderson for assistance developing the HPLC protocol presented here, and E. Brettman for providing GFP-expressing Lgy. We also thank A.C.M. Boon, C.E. Cameron, D. Goldberg, P. Olivo, C. Stallings, and N. Tolia for comments and/or suggestions. Supported by NIH grants R01AI029646 and R56AI099364 to SMB and Sigma-Aldrich Predoctoral and Sondra Schlesinger Graduate Student fellowships to JIR.

4.7 – References


Association of the Endobiont Double-Stranded RNA Virus LRV1 With Treatment Failure for Human Leishmaniasis Caused by *Leishmania braziliensis* in Peru and Bolivia. *J Infect Dis*


Zika Virus Replication and Delays Disease Progression in a Robust Mouse Infection Model. *PLoS Negl Trop Dis* **10**, e0004695


4.8 – Table

Table 4-1: Effect of 2CMA-TP on Lgy LRV1 RDRP activity.

Data represent IC50 values for the inhibition of full-length and small RDRP products. Values are means of 3-4 experiments ±S.D., calculated with Microsoft Excel. IC50 values greater than 600 µM were extrapolated from available data.

<table>
<thead>
<tr>
<th>RDRP Product</th>
<th>Low Density (n=3)</th>
<th>Medium Density (n=3)</th>
<th>High Density (n=4)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full-Length</td>
<td>130 ± 67</td>
<td>260 ± 190</td>
<td>410 ± 140</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Small</td>
<td>510 ± 330</td>
<td>740 ± 400</td>
<td>1000 ± 480</td>
<td>&lt;0.07</td>
</tr>
</tbody>
</table>
Figure 4-1: Life cycle of LRV1 within the Leishmania cytoplasm.

RNAs are indicated in color (+strand blue, -strand red); the dsRNA genome within the mature virion is shown as straight line while ssRNA are shown as jagged lines. The viral RDRP (black trapezoid) is shown fused to a capsid monomer (white circle), reflecting the current theory that the RDRP is generated through frame shift translation (26-28).
Clarified parasite lysates were separated on a CsCl density gradient and the relative amount of viral capsid protein in each fraction was measured. Data for one representative gradient are shown out of the 7 performed. The “peak” fractions of low-, medium- and high-density (LD, MD and HD), which were taken for RDRP assays, are labeled.

*Figure 4-2: Distribution of viral capsid protein across a CsCl density gradient.*
Figure 4-3: Radiolabeled RNAs produced by purified Lgy LRV1 RDRP in vitro.

Panel A. RDRP in LD, MD, and HD fractions (Fig. 2) was assayed using [α-32P]UTP incorporation, as described in “Experimental Procedures.” Radiolabeled RNAs were run alongside pure [α-32P]UTP on a native agarose gel. The full-length and small RDRP products are labeled for reference. Panel B. RDRP reactions were performed in the presence of 0, 10, 30, 100, 300, or 600 µM 2CMA-TP. As a negative control, the RDRP reaction was run using a mock HD fraction isolated from LRV1- Lgy parasites. Native agarose gels of radiolabeled RDRP products showed that RDRP activity decreased with increasing 2CMA-TP concentrations. A representative titration using the HD fraction is shown here.
Figure 4-4: Inhibition of RDRP activity of purified Lgy LRV1 virions by 2CMA-TP.

RDRP reactions were run in the presence of 2CMA-TP and the amounts of full-length and small products were quantified. These amounts were normalized to the amount of product formed in the absence of 2CMA-TP. The averages and SDs (calculated with Microsoft Excel) from three LD virion titrations and four HD virion titrations are shown. MD virions show intermediate profiles (not shown; see Table 1). Effect of 2CMA-TP on production of full-length (Panel A) and short (Panel B) RNAs by RDRP activity in HD (solid line) and LD (dotted line) virions.
Figure 4-5: Specificity of Lgy RDRP inhibition by 2CMA-TP relative to 2CMA and dATP.

RDRP reactions were run for 1 hour in the presence of 1 mM 2CMA, 600 µM dATP, or 600 µM 2CMA-TP. The amount of full-length (left) and small products (right) were measured and normalized to untreated control reactions. The averages and ranges are shown for two experiments.
Figure 4-6: Lgy M4147 LRV1+ parasites synthesize 2CMA-TP.

Panel A. Standards establishing the HPLC elution time of 2CMA-TP relative to dGTP, the exogenous internal standard (although present naturally, the concentrations are far below that added here). The figure shows the HPLC elution profiles of a mixture of ATP and the dGTP internal standard with (black) or without (gray) 2CMA-TP. The small peak eluting in the 2CMA-TP containing experiment between dGTP and ATP is presumed to be 2CMA-DP. Panel B. Detection of 2CMA-TP in Lgy incubated in 10 µM 2CMA for 20 hours. In order to correct for
variation in extraction efficiency and HPLC elution times, all samples have 7 nmol dGTP spiked in immediately prior to extraction. Standards are as in panel A.
Figure 4-7: L. guyanensis parasites accumulate high levels of 2CMA-TP.

Panel A. Graph showing the measured intracellular concentration of 2CMA-TP formed after 18 hr incubation in the indicated concentration of external 2CMA. Intracellular nucleotides were extracted and quantified by HPLC, and concentrations estimated from cell volumes estimated from a standard curve of forward scattering measurements (Experimental Procedures; Fig. S2). For reference, the LRV1 EC50 is marked on the X axis (black arrow) and the minimum RDRP IC50 on the Y axis (grey arrow). The ratio of internal 2CMA-TP to external 2CMA concentrations is plotted in blue. Panel B. Plot of 2CMA-TP accumulation after adding 10 µM 2CMA to late-log-phase cells (n=2). Each point represents a concentration measured from a separate culture.
Figure 4-8: Retention of 2CMA-TP following removal of 2CMA.

LRV1+ Lgy M4147 parasites were incubated for 19 hr in the presence of 10 µM 2CMA; at that time, cells were harvested and resuspended in drug free medium, and intracellular 2CMA-TP levels were measured as described in the legend to Fig. 7 at 2, 4, or 8 hours after removal of drug. Each individual data point (black circles) is the concentration from a single culture. This experiment was repeated twice using two independent cultures per time point (n=4). The averages and standard deviations of each time point are plotted in as black dashed lines. Panel A. Data uncorrected for cell growth. Panel B. Data corrected for cell growth (about 1.4-fold over the course of the experiment).
Figure 4-9: Parameterization and output for Gillespie simulation of LRV1 loss.

Panel A. Results of Gillespie simulation assuming relative inhibition of LRV1 and parasite replication to be 1/1 to 1/4. A theoretical plot for total inhibition of viral replication and ideal viral dilution is shown (gray dotted line), and two experimental data sets from Kuhlmann et al (11) are shown as dark dashed lines. Panel B. RDRP and parasite growth inhibition data relevant to parameterization of the Gillespie simulation. The X axes relate the external 2CMA and intracellular 2CMA-TP concentrations (Fig. 7). The rate of parasite growth in the presence of 2CMA (solid line; △) and the rate of RDRP activity (LD virions making full-length products) in the presence of 2CMA-TP (●) were normalized relative to untreated controls. The best-fit IC50 curve (grey dashed line) was fitted to the RDRP activity data. Assuming that the most drug-sensitive RDRP activity defines the rate of virus replication, the relative inhibition ratio (blue dotted line; ■) was defined as the ratio of RDRP activity to parasite growth.
Figure 4-S1: Structures of LRV1 Inhibitors.

Chemical structures of (A) adenosine, (B) 2’-C-methyladenosine, and (C) 7-deaza-2’-C-methyladenosine.
Figure 4-S2: Relationship Between Parasite Volume and Light Scattering

Relationship between L. guyanensis M4147 parasite volume and forward-scattering intensity at three points in log phase growth. *Leishmania* cells are known to decrease in volume as cell density increases toward stationary phase where they differentiate to smaller metacyclic forms. (Sacks & Perkins, 1984)
Figure 4-S3: Standard curves for HPLC trace area vs. compound amounts.

HPLC chromatogram peak areas correlate linearly with amounts of nucleotide injected.
4.10 – Supplementary Text

4.10.1 – Supplementary Text 1: Conversion of flow cytometric scatter profiles to cellular volumes (Image J implementation)

Image stacks of GFP-expressing *Leishmania* parasites were obtained by spinning-disk confocal microscopy (see main methods for more detail). We implemented a ImageJ script to automatically identify parasites and measure their volumes (1). This script takes the 3D confocal microscopy stack and re-slices it along the x- and y-axes to create two new images. Automatic local thresholds are then calculated for each slice of the x-, y-, and z-stacks using Bernsen’s method (2). Each thresholded stack is then re-sliced to the original orientation and all three stacks are added together. This yields an image stack where the value of each voxel corresponds to the number of dimensions in which it was deemed to be part of a cell. Cell volumes were reliably defined by those voxels identified in 2 or 3 dimensions. Volumes were estimated using the ImageJ 3D Objects Counter with the threshold set to 2 (3). Cells in direct contact were occasionally counted as one object. These were manually identified and corrected by dividing the volume of the combined object by the number of cells within it.

3_Way_Local_Threshold.ijm

// 3_Way_Local_Threshold
// John I Robinson

method = "Bernsen";
radius = "25";

// create working copy of current image
tempTitle = getTitle() + "-thresholded";
run("Duplicate...", "title=" + tempTitle + " duplicate");

setBatchMode(true);
rename(tempTitle);
run("8-bit");

// reslice stack from top and left
run("Reslice [...]", "output=0.270 start=Top avoid");
rename("top");
selectWindow(tempTitle);
run("Reslice [/]...", "output=0.270 start=Left avoid");
rename("left");

// calculate local threshold on each view
selectWindow(tempTitle);
run("Auto Local Threshold", "method=" + method + " radius=" + radius + "
parameter_1=0 parameter_2=0 white stack");
selectWindow("top");
run("Auto Local Threshold", "method=" + method + " radius=" + radius + "
parameter_1=0 parameter_2=0 white stack");
selectWindow("left");
run("Auto Local Threshold", "method=" + method + " radius=" + radius + "
parameter_1=0 parameter_2=0 white stack");

// convert back to standard view
selectWindow("top");
run("Reslice [/]...", "output=0.212 start=Top avoid");
selectWindow("left");
run("Reslice [/]...", "output=0.212 start=Top rotate avoid");

// merge results
selectWindow(tempTitle);
run("Subtract...", "value=254 stack");
selectWindow("Reslice of top");
run("Subtract...", "value=254 stack");
selectWindow("Reslice of left");
run("Subtract...", "value=254 stack");
imageCalculator("Add stack", tempTitle,"Reslice of top");
imageCalculator("Add stack", tempTitle,"Reslice of left");

// apply a useful LUT so we can see the results
reds = newArray(256);
reds[3] = 255;
greens = newArray(256);
greens[1] = 121;
blues = newArray(256);
blues[2] = 255;
setLut(reds, greens, blues);

// clean up
selectWindow("top");
close();
selectWindow("left");
close();
selectWindow("Reslice of top");
close();
selectWindow("Reslice of left");
close();
setBatchMode(false);
print("Done processing " + tempTitle);
selectWindow(tempTitle);
References for Supplementary Text 1:


4.10.2 – Supplementary Text 2: Gillespie simulation of LRV1 loss under 2CMA inhibition and Java implementation

As described in the main text, this simulation models *Leishmania* and LRV1 replication. It utilizes the next-reaction modification (NRM) to the Gillespie algorithm (1). The model implements two kinds of entity: parasites and virions. Each parasite can contain a number of virions. The model also includes two types of process: parasite division and virion replication. The NRM algorithm works by first randomly picking times remaining until each entity completes a process, using a probability distribution defined by that process’ rate. The traditional Gillespie algorithm, which simulates elementary chemical reactions, uses an exponential distribution of waiting times, which is appropriate for Poisson-type processes (2). Here, since cell division and virus replication are complex processes involving many steps, we pick individual event times from Gaussian distributions about the mean parasite doubling and virus replication times (1). The selected waiting times are then sorted in a priority queue. At each step, the process with the shortest waiting time is executed and any dependent processes have their waiting times updated. Given only two processes, this model’s behavior is defined by the relationship between those processes’ rates. For simplicity’s sake, we let the parasite division
time be exactly 8 hours. Given that the number of virions per parasite remains roughly constant, we assume that the effective virion replication rate is equal to the parasite division time under normal conditions (3,4). If the unit of time in this simulation is converted from hours to multiples of the parasite division time, the simulation’s behavior depends entirely on the ratio between the virion replication time and the parasite doubling time.

The secondary parameters defining this model are the initial number of cells and the initial number of LRV1 particles per cell. We found that modeling an initial population of 1000 cells over time, tracking the viral titer in each cell, gave indistinguishable profiles across multiple runs, so we used this value for each simulation. Initial LRV1 numbers were set at 16 per cell, consistent with previous studies (3).

Modeling parasite division required also modeling the distribution of virions between the daughter cells. Previously, we have shown that virions are distributed roughly equally when parasites divide (3). In theory, this would be modeled using a binomial distribution. However, such a mechanism would allot all virions to one daughter cell frequently enough (1 in $6.6 \times 10^5$ divisions with a full 16 virions) to make LRV1+ populations unstable and cause spontaneous virus loss far more often than actually observed (5). Instead, we assumed that virion partitioning is not entirely random, but instead based on location, resulting in daughter cells receiving closer to 50% of the parent’s virions. To model this, the proportion of virions given to new daughter cells was selected from a Gaussian distribution with mean of 50% and standard deviation of 6.25%. This standard deviation was selected to give a vanishingly small chance of stochastically “curing” a daughter of a parasite with 16 virions.

Java implementation

NRSMain2.java
This class includes the overarching logic that controls simulations. Several arrays of parameters allow users to define and run a series of simulations automatically. This program makes use of the Apache Math Library, version 3.6.1.

```java
package nrs;

import java.io.File;
import java.io.FileNotFoundException;
import java.io.PrintStream;
import java.util.HashMap;
import java.util.LinkedList;
import java.util.Map.Entry;
import java.util.PriorityQueue;
import java.util.TreeMap;
import java.util.concurrent.ForkJoinPool;
import java.util.concurrent.TimeUnit;

import org.apache.commons.math3.random.MersenneTwister;
import org.apache.commons.math3.special.Erf;

public class NRSMain2 {
    private static MersenneTwister RAND = new MersenneTwister();
    private static double SD_SCALE = 16;
    private static double DEFAULT_CELL_T2 = 8;
    private static double DEFAULT_VIRUS_T2 = 8;
    /
    Number of parasites to put into each simulation initially.
    */
    private static int POPULATION_SIZE = 1000;
    /**
     * How many hours to run the simulation for.
     */
    private static double STOP_TIME = 48;
    /**
     * How many intermediate population snapshots to record.
     */
    private static double SNAP_INTERVAL = 8;

    /**
     * @param args
     */
    public static void main(String[] args) {
        Parasite newCell;
        Virion vir;
        Simulation sim;
        LinkedList<Simulation> sims;
        PriorityQueue<Process> equilPop, popClone;
    }
}
```
// Set parameters for the simulations we'll be running.  
// Each column corresponds to the parameters for one simulation.

// Parasite doubling times
double[] cellT2s = {8,8,8,8,8,8};
// Virus replication times
double[] virusT2s = { 8*2, 8*2, 8*2, 8*3, 8*3, 8*3};
// How many virions per cell initially
int[] initVirions = { 16, 16, 16, 16, 16, 16};
// Limit on the number of virions per cell. Keep this >>16.
int[] maxVirions = { 80, 80, 80, 80, 80, 80};

// create initial population
PriorityQueue<Process> equilPop = new PriorityQueue<>();
int startPop = POPULATION_SIZE;
for(int k = 0; k < startPop; k++)
{
    // we'll handle the virions belonging to each parasite here too
    Parasite newCell = new Parasite(NRSMain2.initialTau(DEFAULT_CELL_T2,
            DEFAULT_CELL_T2/SD_SCALE));
    // clone all the virions
    for(int j = 0; j < initVirions[0]; j++)
    {
        // we're messing with virus T2, so we need to recalculate tau
        Virion vir = new Virion(newCell,  
            NRSMain2.initialTau(DEFAULT_VIRUS_T2,
            DEFAULT_VIRUS_T2/SD_SCALE));
        newCell.addVirion(vir);
        equilPop.add(vir);
    }
    equilPop.add(newCell);
}

// set up thread pool to handle all the simulations concurrently
ForkJoinPool threadPool = new ForkJoinPool(Runtime.getRuntime().availableProcessors() - 1);

// now run the actual simulations
LinkedList<Process> sims = new LinkedList<>();
for(int i = 0; i < maxVirions.length; i++)
{
    // Clone each parasite and virion
    PriorityQueue<Process> popClone = new PriorityQueue<>();
    for(Process p : equilPop)
    {
        if(p instanceof Parasite)
        {
            // copy constructor clones the virions too
            newCell = new Parasite((Parasite)p);
            popClone.add(newCell);
            popClone.addAll(newCell.getVirionsView());
            // need to update tau values to reflect new rates
            newCell.setTau(NRSMain2.initialTau(cellT2s[i],
                cellT2s[i]/SD_SCALE));
        }
    }
    sims.add(new Cell(newCell, popClone));
}

// now run the simulations in parallel
for(Process p : sims)
    threadPool.execute(p);
for (Virion v : newCell.getVirionsView())
{
    v.setTau(NRSMain2.initialTau(virusT2s[i],
    virusT2s[i]/SD_SCALE));
}
}
sim = new Simulation(popClone, cellT2s[i], virusT2s[i],
maxVirions[i],
    STOP_TIME, SNAP_INTERVAL);
sims.add(sim);
threadPool.execute(sim);

// wait for all the simulations to end
try {
    threadPool.shutdown();
    threadPool.awaitTermination(Long.MAX_VALUE, TimeUnit.DAYS);
} catch (InterruptedException e1) {
    e1.printStackTrace();
}
System.out.println("Done!");
System.out.println(sims.getFirst().getSnapshots());

// print out the data to files
for (int i = 0; i < sims.size(); i++)
{
    try {
        PrintStream ps = new PrintStream(new File("sim-" + i + ".csv")));
        printSimulation(sims.get(i), ps);
        ps.close();
    } catch (FileNotFoundException e) {
        System.err.println("Failed to open file: sim-" + i + ".csv");
        e.printStackTrace();
    }
}

/**
 * Pick a random tau value for a process, assuming that instances of
 * that
 * process are at steady state.
 * @param mu the mean tau value
 * @param sigma the standard deviation of tau values
 * @return the random tau value
 */
private static double initialTau(double mu, double sigma)
{
    double a = mu/(sigma*Math.sqrt(2));
    double b = (1+Erf.erf(a))*Math.PI*Math.exp(a*a);
    double slope = b/(mu*b + sigma*Math.sqrt(2*Math.PI));
    return RAND.nextDouble()/slope;
}

private static void printSimulation(Simulation sim, PrintStream ps)
HashMap<Integer, Long> snap;
long count, sum;
double gSum, time;
StringBuilder hdr, line;

// get maximum number of virions allowed in the simulation
int maxVirions = sim.getMaxVirions();

// get the list of snapshots
TreeMap<Double, HashMap<Integer, Long>> snaps = sim.getSnapshots();

// build header and write it
hdr = new StringBuilder("Time,");
for(int i = 0; i <= maxVirions; i++)
{
    hdr.append(i);
    hdr.append(',');
}
hdr.append("Total,GeoMean");
ps.println(hdr);

line = new StringBuilder();

// loop over all the snapshots
for(Entry<Double, HashMap<Integer, Long>> entry : snaps.entrySet())
{
    time = entry.getKey();
    // write out the time stamp
    line.append(time);
    line.append(',');

    // write out the parasite counts
    snap = entry.getValue();
    sum = 0;
gSum = 0;
    for(int i = 0; i <= maxVirions; i++)
    {
        if(snap.containsKey(i))
        {
            count = snap.get(i);
        }
        else
        {
            count = 0;
        }
        sum += count;
gSum += count*Math.log(i+1);
        line.append(count);
        line.append(',');
    }
    // write out total and geometric mean (Williams' modification)
    line.append(sum);
    line.append(',');
gSum = Math.exp(gSum/sum) - 1;
line.append(gSum);
// write the line
ps.println(line);
// reset the line
line.setLength(0);
}
Simulation.java

This class implements an individual LRV1 simulation, given an initial parasite population and a set of parameters and options.

```java
package nrs;

import java.util.HashMap;
import java.util.HashSet;
import java.util.LinkedList;
import java.util.Map.Entry;
import java.util.PriorityQueue;
import java.util.TreeMap;
import java.util.concurrent.ForkJoinTask;
import java.util.concurrent.RecursiveAction;

import org.apache.commons.math3.distribution.NormalDistribution;
import org.apache.commons.math3.random.MersenneTwister;

public class Simulation extends RecursiveAction {
    private static final long serialVersionUID = -2016479837671294476L;
    private static final double SD_SCALE = 16;
    // Narrows the standard deviation of the proportion of virions given to a new daughter cell.
    private static final double DIVISION_SD_SCALE = 2;
    private MersenneTwister myRand;
    private double myCellT2, myVirusT2;
    private int myMaxVirions;
    private double myStopTime, mySnapInterval;
    private NormalDistribution myCellPDF, myVirusPDF;
    private TreeMap<Double, HashMap<Integer, Long>> mySnapshots;
    private PriorityQueue<Process> myPopulation;

    public Simulation(PriorityQueue<Process> population, double cellT2, double virusT2, int maxVirions, double stopTime, double snapInterval) {
        myPopulation = population;
        myCellT2 = cellT2;
        myVirusT2 = virusT2;
        myMaxVirions = maxVirions;
        myStopTime = stopTime;
        mySnapInterval = snapInterval;

        myRand = new MersenneTwister();
        myCellPDF = new NormalDistribution(myRand, myCellT2, myCellT2/25.74);
        myVirusPDF = new NormalDistribution(myRand, myVirusT2, myVirusT2/25.74);
```
mySnapshots = new TreeMap<>();

/**
 * Get a map of the snapshots taken by this simulation.
 * @return the snapshots
 */
public TreeMap<Double, HashMap<Integer, Long>> getSnapshots() {
    return mySnapshots;
}

/**
 * @return the maxVirions
 */
public int getMaxVirions() {
    return myMaxVirions;
}

private void run() {
    double time, mean, sd;
    int numVirions, d1, d2;
    LinkedList<Double> snapTimes;
    Double nextSnap;
    Process rxn;
    Parasite cell, daughter;
    Virion vir, newVir;

    // list of processes with changed tau values
    HashSet<Process> needsUpdating = new HashSet<>();

    // calculate the list of snapshot times
    snapTimes = new LinkedList<Double>();
    for(double i = 0; i <= myStopTime; i += mySnapInterval)
    {
        snapTimes.add(i);
    }
    nextSnap = snapTimes.pop();

    time = 0;

    // take an initial snapshot
    System.out.println("Running simulation: " + this);
    mySnapshots.put(nextSnap, this.takeSnapshot());
    nextSnap = snapTimes.pop();

    // Any time a parasite divides or virion replicates:
    // Generate tau for new cells or virions
    // Put tau values into the priority queue
    // Tau for next event is minimum value in queue
    while(time < myStopTime)
    {
        // (#) Pick reaction from P with minimum tau_r
        rxn = myPopulation.peek();
        // Update time
        time = rxn.getTau();
        // Change numbers of molecules to reflect the reaction
        if(rxn instanceof Parasite)
// parasite is dividing
cell = (Parasite)rxn;
numVirions = cell.getVirionCount();
// use squashed normal distribution
mean = numVirions/2.0;
sd = Math.sqrt(mean)/2.0/DIVISION_SD_SCALE;
d1 = (int) Math.round(sd*myRand.nextGaussian() +
mean);
if(d1 < 0)
{
    d1 = 0;
}
else if(d1 >= numVirions)
{
    d1 = numVirions;
}
d2 = numVirions - d1;
// if the dividing parasite was at maximum virions,
// we need to
// restart virus replication
if(numVirions >= myMaxVirions)
{
    for(Virion v : cell.getVirionsView())
    {
        v.setTau(time + this.pickVirusTau());
        needsUpdating.add(v);
    }
}
// create and populate daughter cell
daughter = new Parasite(time +
this.pickParasiteTau());
needsUpdating.add(daughter);
for(int i = 0; i < d2; i++)
{
    vir = cell.removeVirion(0);
    vir.setHost(daughter);
    daughter.addVirion(vir);
}
// pick new tau value for the parent cell
cell.setTau(time + this.pickParasiteTau());
needsUpdating.add(cell);

} else if(rxn instanceof Virion)
{
    // virus is replicating
    vir = (Virion)rxn;
    cell = vir.getHost();
    // create new virion
    newVir = new Virion(cell, 0);
    cell.addVirion(newVir);
    if(cell.getVirionCount() >= myMaxVirions)
    {
        // maximum number of virions reached, so stop
        replication
        for(Virion v : cell.getVirionsView())
        {
v.setTau(Double.POSITIVE_INFINITY);
needsUpdating.add(v);
}

else
{
    // set tau for new virion and one that
    // replicated
    vir.setTau(time + this.pickVirusTau());
    needsUpdating.add(vir);
    newVir.setTau(time + this.pickVirusTau());
    needsUpdating.add(newVir);
}

// Rebuild tau queue to account for changes
myPopulation.removeAll(needsUpdating);
myPopulation.addAll(needsUpdating);
needsUpdating.clear();

// take a snapshot, if applicable
if(time >= nextSnap)
{
    mySnapshots.put(nextSnap, this.takeSnapshot());
    nextSnap = snapTimes.poll();
}

// Loop back to (#)

/**
 * Select a random length of time that a parasite will wait before dividing.
 * @return the time
 */
private double pickParasiteTau()
{
    return myCellPDF.sample();
}

/**
 * Select a random length of time that a parasite will wait before dividing.
 * @return the time
 */
private double pickVirusTau()
{
    return myVirusPDF.sample();
}

/**
 * Takes a snapshot of the current parasite population.
 * @return a map with the number of parasites containing a given number
 * of virions
 */
private HashMap<Integer, Long> takeSnapshot();
```java
{    int numVirions;
    Long count;
    HashMap<Integer, Long> histo = new HashMap<>();

    for(Process p : myPopulation)
    {
        if(p instanceof Parasite)
        {
            // increment the bin for parasites with this many virions
            numVirions = ((Parasite)p).getVirionCount();
            count = histo.get(numVirions);
            if(count == null)
                histo.put(numVirions, 1L);
            else
                histo.put(numVirions, count + 1L);
        }
    }
    return histo;
}

@Override
protected void compute() {
    int index;
    Parasite cell;
    Simulation sim1, sim2;
    PriorityQueue<Process> pop1, pop2;

    if(myPopulation.size() > 200)
    {
        // this simulation is too big, so split it up by turning it into
        // two jobs with half as many parasites
        index = 0;
        pop1 = new PriorityQueue<>();
        pop2 = new PriorityQueue<>();
        for(Process p : myPopulation)
        {
            if(p instanceof Parasite)
            {
                cell = (Parasite)p;
                // add parasite and its virions to one population or the other
                if(index%2 == 0)
                {
                    pop1.add(cell);
                    pop1.addAll(cell.getVirionsView());
                }
                else
                {
                    pop2.add(cell);
                    pop2.addAll(cell.getVirionsView());
                }
                index++;
            }
        }
    }
}
```
sim1 = new Simulation(pop1, myCellT2, myVirusT2,
myMaxVirions,
    myStopTime, mySnapInterval);
sim2 = new Simulation(pop2, myCellT2, myVirusT2,
myMaxVirions,
    myStopTime, mySnapInterval);
// launch the two simulations and wait for them to complete
ForkJoinTask.invokeAll(sim1, sim2);
this.combine(sim1, sim2);
} else {
    // we've got a small enough simulation, so just run it
    this.run();
}
/**
 * Merges two sub-simulations back into this one.
 * @param sim1
 * @param sim2
 */
private void combine(Simulation sim1, Simulation sim2) {
    long count;
    Integer numVirions;
    Parasite cell;
    HashMap<Integer, Long> histo1, histo2;
    // clear the out-of-date population
    myPopulation.clear();
    // add each sub-population
    for(Process p : sim1.myPopulation)
    {
        if(p instanceof Parasite)
        {
            cell = (Parasite)p;
            myPopulation.add(cell);
            myPopulation.addAll(cell.getVirionsView());
        }
    }
    for(Process p : sim2.myPopulation)
    {
        if(p instanceof Parasite)
        {
            cell = (Parasite)p;
            myPopulation.add(cell);
            myPopulation.addAll(cell.getVirionsView());
        }
    }
    // combine snapshots
    for(Entry<Double, HashMap<Integer, Long>> e :
sim1.getSnapshots().entrySet())
    {
        if(!mySnapshots.containsKey(e.getKey()))
        {
            // this snapshot is not present in the parent list
            }
mySnapshots.put(e.getKey(), e.getValue());
}
else
{
    histo1 = mySnapshots.get(e.getKey());
    histo2 = e.getValue();
    for(Entry<Integer, Long> eh : histo2.entrySet())
    {
        numVirions = eh.getKey();
        if(histo1.containsKey(numVirions))
        {
            // add this entry to a preexisting one
            count = histo1.get(numVirions);
            count += histo2.get(numVirions);
            histo1.put(numVirions, count);
        }
        else
        {
            // add a new entry
            histo1.put(numVirions, eh.getValue());
        }
    }
}
for(Entry<Double, HashMap<Integer, Long>> e : sim2.getSnapshots().entrySet())
{
    if(!mySnapshots.containsKey(e.getKey()))
    {
        // this snapshot is not present in the parent list
        mySnapshots.put(e.getKey(), e.getValue());
    }
    else
    {
        histo1 = mySnapshots.get(e.getKey());
        histo2 = e.getValue();
        for(Entry<Integer, Long> eh : histo2.entrySet())
        {
            numVirions = eh.getKey();
            if(histo1.containsKey(numVirions))
            {
                // add this entry to a preexisting one
                count = histo1.get(numVirions);
                count += histo2.get(numVirions);
                histo1.put(numVirions, count);
            }
            else
            {
                // add a new entry
                histo1.put(numVirions, eh.getValue());
            }
        }
    }
}

/* (non-Javadoc)
* @see java.lang.Object#toString()
 */

@Override
public String toString() {
    return "Simulation [cellT2=" + myCellT2 + ", virusT2=" + myVirusT2 + ", maxVirions=" + myMaxVirions + ", stopTime=" + myStopTime + ", processes=" + myPopulation.size() + "]";
}
Parasite.java

This class defines the properties of a parasite. It holds the time when this parasite will divide and also keeps track of the virions contained within this parasite.

```java
package nrs;

import java.util.Collection;
import java.util.Collections;
import java.util.LinkedList;
import java.util.List;

public class Parasite extends Process {
    private LinkedList<Virion> myVirions;

    public Parasite() {
        this(Double.POSITIVE_INFINITY, Collections.<Virion> emptyList());
    }

    public Parasite(double tau) {
        this(tau, Collections.<Virion> emptyList());
    }

    public Parasite(double tau, Collection<Virion> virions) {
        super(tau);
        myVirions = new LinkedList<Virion>(virions);
        // make sure all virions are assigned to me
        for(Virion v : myVirions) {
            v.setHost(this);
        }
    }

    public Parasite(Parasite p) {
        super(p.getTau());
        myVirions = new LinkedList<Virion>();
        Virion copy;
        for(Virion v : p.myVirions) {
            myVirions.add(v);
        }
    }
    /**
     * Copy constructor for parasites.
     * @param p the parasite to copy
     */
    public Parasite(Parasite p) {
        super(p.getTau());
        myVirions = new LinkedList<Virion>();
        Virion copy;
        for(Virion v : p.myVirions) {
            myVirions.add(v);
        }
    }

    /*
     * @param tau when this parasite will divide
     * @param virions the virions inside this parasite
     */
    public Parasite(double tau, Collection<Virion> virions) {
        super(tau);
        myVirions = new LinkedList<Virion>(virions);
        // make sure all virions are assigned to me
        for(Virion v : myVirions) {
            v.setHost(this);
        }
    }
```
copy = new Virion(v);
copy.setHost(this); // set the cloned host
myVirions.add(copy);
}

/**
 * @return the virions
 */
public List<Virion> getVirionsView() {
    return Collections.unmodifiableList(myVirions);
}

/**
 * @return
 * @see java.util.LinkedList#size()
 */
public int getVirionCount() {
    return myVirions.size();
}

/**
 * @param e
 * @return
 * @see java.util.LinkedList#add(java.lang.Object)
 */
public boolean addVirion(Virion e) {
    return myVirions.add(e);
}

public Virion removeVirion(int index) {
    return myVirions.remove(index);
}

/**
 * Simply calls the Process equals method, which compares objects based on
 * their tau values.
 * @param obj the other process
 */
@Override
public boolean equals(Object obj) {
    return super.equals(obj);
}

/* (non-Javadoc)
 * @see nrs.Process#hashCode()
 */
@override
public int hashCode() {
    return super.hashCode();
}
Virion.java

This class implements the properties of a virion. It keeps track of when this virion will next replicate and what parasite it is infecting.

```java
package nrs;

public class Virion extends Process {
    private Parasite myHost;

    public Virion(Parasite host, double tau) {
        super(tau);
        myHost = host;
    }

    /**
     * Copy constructor for virions.
     * @param v the virion to copy
     */
    public Virion(Virion v) {
        super(v.getTau());
        this.myHost = v.myHost;
    }

    /**
     * @return the host
     */
    public Parasite getHost() {
        return myHost;
    }

    /**
     * @param host the host to set
     */
    public void setHost(Parasite host) {
        myHost = host;
    }

    /**
     * Simply calls the Process equals method, which compares objects based on
     * their tau values.
     * @param obj the other process
     */
    @Override
    public boolean equals(Object obj) {
        return super.equals(obj);
    }
}
```
/* (non-Javadoc)
 * @see nrs.Process#hashCode()
 */

@Override
public int hashCode() {
    return super.hashCode();
}
}
This generic class defines processes in a next-reaction Gillespie simulation. The Parasite and Virion classes represent cell division and virus replication processes, so they inherit from this class.

```java
package nrs;

/**
 * Generic class defining processes in a next-reaction Gillespie simulation.
 */
public class Process implements Comparable<Process> {
    private double myTau;

    public Process(double tau)
    {
        myTau = tau;
    }

    /**
     * Gets the absolute time when this process will next occur.
     * @return the time
     */
    public double getTau()
    {
        return myTau;
    }

    /**
     * Set the absolute time when this process will next occur.
     * @param tau the time
     */
    public void setTau(double tau)
    {
        myTau = tau;
    }

    /* (non-Javadoc)
     * @see java.lang.Comparable#compareTo(java.lang.Object)
     */
    @Override
    public int compareTo(Process o) {
        return Double.compare(this.myTau, o.myTau);
    }

    /* (non-Javadoc)
     * @see java.lang.Object#hashCode()
     */
    @Override
    public int hashCode() {
        final int prime = 31;
        final int result = prime * (int) myTau;
        return result;
    }
}
```
int result = 1;
long temp;
temp = Double.doubleToLongBits(myTau);
result = prime * result + (int) (temp ^ (temp >>> 32));
return result;
}

/* (non-Javadoc)
 * @see java.lang.Object#equals(java.lang.Object)
 * @Override
public boolean equals(Object obj) {
    if (this == obj)
        return true;
    if (obj == null)
        return false;
    if (!(obj instanceof Process))
        return false;
    Process other = (Process) obj;
    if (Double.doubleToLongBits(myTau) != Double.doubleToLongBits(other.myTau))
        return false;
    return true;
}

References for Supplementary Text 2:


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Chapter 5: Conclusions and Future Directions
Preface

The first draft of this chapter was written by JIR. The final version presented here incorporates comments from SMB.
5.1 – Project Goals

Given that *Leishmania* RNA Virus 1 (LRV1) exacerbates the severity of leishmaniasis in both mouse models and human disease (Ives et al. 2011; Hartley et al. 2016; Adaui et al. 2015; Ito et al. 2015; Cantanhede et al. 2015; Bourreau et al. 2015), tools for detecting and eliminating LRV1 could prove useful in treating human leishmaniasis. These tools would also be useful in the laboratory to probe the relationship between *Leishmania* parasites, LRV1, and the host immune system. Until recently, detection of LRV1 has been limited to time- and labor-intensive techniques not conducive to use in a clinical or resource-limited setting. (Widmer et al. 1989) Furthermore, with a single serendipitous exception, (Ro et al. 1997) it had been impossible to eliminate LRV1 from infected parasites. The first portion of this work, Chapter 2, describes a toolkit of methods for detecting and quantifying LRV1, with an emphasis on simple, relatively rapid techniques that could be adapted for clinical use. The greater portion of this work focuses on the discovery of effective LRV1 inhibitors (Chapter 3) and the investigation of their mode of action (Chapter 4). Finally, Appendix B lays the groundwork for attempting to moderate the immune effects of LRV1 by inhibiting it in a mouse model of leishmaniasis. Appendix A describes a drug screening setup for efficiently identifying *Leishmania* inhibitors, LRV1 inhibitors, and *Leishbunyavirus* inhibitors at the same time. In this chapter, I will summarize my findings, discuss their wider implications, and propose future work to expand upon them.

5.2 – Development of Tools for the Detection of LRV1: Techniques and Uses

In Chapter 2, we set out to collect or develop a wide range of tools for detecting, quantifying, and studying LRV1. These began with the “traditional” technique of detecting the dsRNA viral genome by purifying total parasite RNA and running it on a gel. (Tarr et al. 1988; Widmer et al. 1989) This method, although sequence-agnostic and useful for identifying new
viruses, is also time-consuming and requires specialized laboratory apparatus. Quantitative PCR of reverse transcribed viral RNA is a close relative of this method. It too requires significant time and resources, but also depends upon the precise sequences of the particular virus in question. Alternately, an antibody raised against the LRV1 capsid can also be used to detect LRV1. (Cadd et al. 1993) Although in this work the antibody was exclusively visualized by immunofluorescence imaging, for our drug screening efforts in Chapter 3 we modified the procedure for analysis by flow cytometry, effectively performing the same analysis over larger numbers of parasites. The capsid antibody, however, is limited to strains of LRV1 that share the epitopes it recognizes. In fact, although the antibody was raised against the LRV1 from an L. guyanensis strain, it was unable to recognize the virus from another L. guyanensis strain. The most promising approaches utilized an antibody that recognizes double-stranded RNA. (Bonin et al. 2000) This effectively allowed us to combine the efficiency of the capsid-based approach with the sequence independence of the RNA band approach. Importantly, the antibody was able to selectively stain LRV1+ parasites immobilized on a nitrocellulose membrane. Such assays are ideal for adaptation to clinical or field work, particularly since they were effective on samples taken directly from mouse lesions. I believe that with relatively little development cost these blotting methods could be converted into easy-to-use diagnostics that would enable healthcare workers to tailor leishmaniasis treatment depending on the presence or absence of LRV1.

In the laboratory, antibody tools have proved highly useful for my research. As previously mentioned, we adapted the anti-capsid antibody for use in a flow cytometer, which allowed us to rapidly screen for LRV1 inhibitors by monitoring changes in average LRV1 burden. I also utilized the anti-capsid antibody with the membrane blotting technique as a rapid way to locate virions in density gradient fractions as I optimized my virus purification protocol.
5.3 The Impact of LRV1 on Leishmaniasis and Approaches to Target it

Studies of the role of LRV1 in disease and parasite biology were initially limited by the stability and non-infectivity of LRV1. Controlled comparisons of LRV+ and LRV- parasites were limited to a single isogenic pair of LRV+ and LRV- parasite strains generated through the fortuitous loss of LRV1 in a *Leishmania guyanensis* strain. (Ro et al. 1997) Recently, our lab leveraged the RNAi pathway present in the *Leishmania (Viannia)* sub-genus to knock down and eventually eliminate LRV1. (Lye et al. 2010; Brettmann et al. 2016) However, this technique requires genetic manipulation of the parasite, which makes it a slow and laborious way to eliminate the virus. Furthermore, RNA interference constructs are limited to targets with well-conserved sequences, so an individual construct would not be applicable to more than a few virus strains.

To remove this bottleneck and work towards clinically-relevant treatments for LRV1, we set out to identify small molecule inhibitors of LRV1 (Chapter 3). Given our relatively limited screening capacity, we sought to assemble a small library of compounds with a high likelihood of activity. Since polymerases are an essentially ubiquitous feature of viruses, nucleoside analogs compose a large proportion of anti-viral compounds. We hoped that an already well-characterized anti-viral compound could be repurposed to inhibit LRV1 in human leishmaniasis. In addition, *Leishmania* parasites are purine auxotrophs (Carter et al. 2008), and thus avidly scavenge all natural nucleobases and nucleosides, plus a number of purine analogs such as allopurinol, increasing the likelihood of identifying a successful nucleoside analog inhibitor of LRV1. (Marr 1983; LaFon et al. 1985) As a result, we selected a number of known anti-viral nucleoside analogs and a wide range of other nucleoside analogs as the core of our screen. Other members of the viral family Totiviridae can be eliminated by growth cycle arrest, so we also
tested compounds like cyclohexamide. (Bhatti et al. 2011; Fink and Styles 1972) Finally, we included protease inhibitors because previous reports had proposed a role for a parasite cysteine protease in the maturation of LRV1 virions. (Carrion et al. 2003)

Out of this screen of approximately 80 compounds, we identified two closely-related inhibitors of LRV1. These were 2’-C-methyl-adenosine (2CMA) and its 7-deaza analog (7d2CMA). Both were relatively potent inhibitors of LRV1 replication, with EC50 concentrations of 3 and 5 µM, respectively. By using moderate concentrations of 2CMA to reduce LRV1 levels, we were able to isolate drug-treated Leishmania clones either with or without LRV1. We utilized these strains to confirm our previous findings that LRV1-infected parasites induce greater pathology and parasite burden than uninfected parasites. Since both compounds are purine analogs, we hypothesized that they were taken up by the purine salvage pathway and converted to nucleotides, which would then inhibit the LRV1 RNA-dependent RNA polymerase (RDRP).

5.4 – Insights into the LRV1 RDRP via its Inhibition by 2CMA Triphosphate

In Chapter 4, I investigated the mechanism of action of 2CMA. Because 2CMA and 7d2CMA are adenosine analogs, I hypothesized that the drug triphosphates would inhibit the LRV1 RDRP. This was supported by prior work done on both of these compounds that identified them as inhibitors of the Hepatitis C Virus (HCV) RDRP. (Carroll et al. 2003; Olsen et al. 2004) In addition, in Chapter 3 I used the PHYRE2 protein structure prediction server to build a tentative three-dimensional model of the LRV1 RDRP. (Kelley and Sternberg 2009) This structure, including the active site, was highly similar to known HCV RDRP structures, which further suggested that the drugs would bind the RDRP. Theoretically, 2CMA could inhibit LRV1 in a similar mode of action to ribavirin by being incorporated into the viral genome and then
inducing fatally-large numbers of mutations. (Cameron and Castro 2001) However, treating parasites with 100 µM 2CMA resulted in complete inhibition of LRV1 replication, which we would expect if a critical viral enzyme like the RDRP were inhibited, but not if the virus was simply accumulating mutations.

Running radiolabeled RDRP products out on an agarose gel revealed the presence of two distinct products: a discrete band the size of the viral genome and a continuum of 100-500 base-pair small products (Chapter 4). Based on their small size and preliminary data suggesting that they are single-stranded, I believe that the small products represent abortive transcripts produced by the RDRP in the context of an in vitro assay. In a similar vein, the full-length product appears to be dsRNA and likely represents the replicase activity of the RDRP. Intriguingly, production of full-length RNA is more sensitive to 2CMA triphosphate (2CMA-TP) than the short products. In purifying LRV1 virions on density gradients, I was able to partially separate three populations of virion with characteristic densities. The lightest population (low-density virions) had a density suggesting that the virions were mostly empty of viral RNA, with the remainder containing mostly immature ssRNA viral genomes. Thus, in the viral RDRP assays, these virions would be largely replicating their genomes, rather than making viral transcripts. Interestingly, the low-density virions were the most sensitive to 2CMA-TP. These results suggest that the RDRP replicase activity may be more sensitive to 2CMA-TP, likely due to some structural rearrangement of the active site to switch from replicating ssRNA to transcribing ssRNA from a dsRNA template. This hypothesis could be tested by purifying the LRV1 RDRP and measuring its activity in the presence of 2CMA-TP and synthetic ssRNA or dsRNA substrates.
5.5 – Mechanism of Action of 2CMA

One of the useful features of 2CMA and 7d2CMA is that they are much more potent against LRV1 than against the parasite. I believe that this difference is the result of the decreased fidelity and increased promiscuity of viral RNA polymerases relative to cellular RNA polymerases. This is a common paradigm that has acquired great importance due to HCV, which has a particularly low-fidelity polymerase. (Moustafa et al. 2014; Castro et al. 2005; Cameron and Castro 2001) In this model, increased promiscuity would allow the LRV1 RDRP to accept non-natural nucleotides that the host RNA polymerase would reject. One piece of supporting evidence is the ease with which I identified compounds with moderate activity when activated with thymidine kinase (TK, Appendix A). Although one compound, 2’-C-methyl-cytidine, was similar to 2CMA, the other compounds had markedly different structures. This suggests that the LRV1 RDRP will accept a range of nucleoside analogs, although it is worth noting that most active compounds are modified on the ribose moiety, not the nucleobase.

5.6– Metabolism of 2CMA and its Significance for Drug Potency

In Chapter 4, I demonstrate that 2CMA-TP inhibits the LRV1 RDRP, but its potency is much lower than expected, with IC50 concentrations from 130 to over 600 µM compared to 2CMA and its 3 µM EC50 in parasites in cell culture. I eliminated the possibility that this was non-specific competition between a non-substrate nucleotide and a natural nucleotide by treating purified LRV1 virions with high concentrations of 2’-deoxy-adesosine triphosphate (dATP). Despite being modified at the 2’-C position, dATP did not inhibit the viral RDRP.

To reconcile the apparent discrepancy between the IC50 of 2CMA-TP in vitro and the EC50 of 2CMA in vivo, I measured the intracellular concentrations of 2CMA-TP. In order to do this, I developed a protocol for extracting and quantifying the nucleotide pools of Leishmania
cells as well as a facile technique for measuring the volume of live parasites, which allowed me to calculate the effective concentrations of 2CMA-TP within cells. These are detailed in Chapter 4. These experiments demonstrated that the low EC50 is achieved because the parasites accumulate high intracellular concentrations of 2CMA-TP. This phenomenon can be easily explained by *Leishmania*’s ability to scavenge nucleosides.

Unexpectedly, high concentrations of adenine in the parasites’ medium did not significantly compete with LRV1 inhibition by 2CMA. Furthermore, in an unpublished experiment I showed that adenosine does not compete with 2CMA either. I believe these results show that adenosine and 2CMA are salvaged via different pathways. Unlike the other purine nucleosides, adenosine can be processed in two ways. Prior work has shown that the majority of adenosine is hydrolyzed to adenine and then converted to hypoxanthine by adenosine aminohydrolase. (Boitz and Ullman 2013; Boitz et al. 2012; Carter et al. 2008) However, *Leishmania* also express an adenosine kinase protein that can directly phosphorylate adenosine to adenosine monophosphate. (Datta et al. 1987; Bhaumik and Datta 1988) Because 2CMA and 7d2CMA are only modified on the sugar moiety, they would only retain activity if phosphorylated directly by adenosine kinase. Thus, the excess adenosine and adenine would be processed independently of any 2CMA or 7d2CMA.

### 5.7– Simulating LRV1 Inhibition Reconciles 2CMA and 2CMA-TP Potency

In order to more clearly demonstrate that LRV1 inhibition in parasites can be explained by LRV1 RDRP inhibition by high intracellular 2CMA-TP concentrations, I developed a computational model of parasite and LRV1 replication (Chapter 4). The parasite population in the model starts with exactly 16 virions per cell, and the parasites and viruses are allowed to
replicate. By changing the relative replication rates of parasites and viruses, I can reproduce much of the behavior of LRV1 in our studies of 2CMA.

This model indirectly illustrates the importance of the mechanisms that limit the number of LRV1 virions per parasite. Prior work has shown that the number of virions per parasite is regulated (Weeks et al. 1992), and that this regulation is not accomplished via the parasite RNA interference pathway. (Brettmann et al. 2016) In the absence of a detailed mechanistic understanding of this regulation, I explored various methods for simulating it. The fact that LRV1 titer slowly rebounded after being partially eliminated with 2CMA (Chapter 3) demonstrates that under some circumstances, LRV1 can replicate faster than the parasites. However, setting the virus replication time shorter than the parasite doubling time caused viral titer to increase exponentially toward infinity. Simply preventing viral replication past an arbitrary maximum number of virions generated unnatural distributions of virions within the simulated parasite population. In this situation, the virus replicates up to the maximum number before the parasite divides again, leading to an artificially large proportion of parasites with the maximum viral titer, plus a significant population of recently divided parasites with half the maximum number. This does not match with the roughly Gaussian distributions of LRV1 titers we observe via flow cytometry (Chapter 3). In addition, the geometric mean of virion number per parasite in such simulations is significantly less than the maximum allowed virion number. Thus, in order to match the average viral titer observed in vivo, the maximum number of virions must be increased. This yields an even less natural result, where the proportion of parasites with the mean viral titer is small, unlike in vivo where the mean and mode closely coincide.

Another possibility, initially proposed by Weeks et al., is that the virus and parasite replication rates are linked under normal conditions. (Weeks et al. 1992) For the purposes of my
model, this would cause the effective parasite and virus replication rates to be equal. Doing so causes the average number of virions per cell to remain stable without a cap on virus titer. In addition, the distribution of virion numbers becomes roughly Gaussian and centers on the average number. Under this hypothesis, treating parasites with 2CMA eliminates LRV1 by uncoupling the rate of virus replication from the rate of parasite replication.

In Chapter 3, we showed that LRV1 is lost by random dilution when completely inhibited by 2CMA. This indicates that each daughter cell receives on average 50% of the virions in its parent. In the simulation, parasite division was originally handled by randomly assigning each of the mother cell’s virions to one of the daughter cells, a process equivalent to using a binomial distribution to choose the number of virions for each cell. However, this method led to the accumulation of parasites without virus, even without inhibiting viral replication. These “cured” parasites arose when a parasite divided and all the virions were assigned to one daughter cell. For a parasite with 16 virions, this has a 1 in $6.6 \times 10^5$ chance of occurring, but with fewer virions, the probability increases rapidly. Given that a standard liquid culture of parasites replicates from $1 \times 10^6$ cells to $\sim 2.5 \times 10^8$ cells in 48 hours, assigning virions randomly would “cure” at least 3800 cells, and these would continue to replicate as well. Thus, a purely random assignment scheme would slowly “cure” an increasing proportion of the parasites until the virus was lost entirely.

On the other hand, the parasites could hypothetically utilize some active method to assign exactly 50% of the virions to each daughter upon division. This avoids the problem of randomly “curing” parasites, but does not fit the data presented in Chapter 3. When parasites were treated with sufficient 2CMA to block LRV1 replication completely, removing 2CMA after 3 rounds of cell doublings revealed that 31% of parasites had entirely lost the virus. Assuming perfect 50% virion assignment, none of these parasites should have been cured. Instead, each would contain
\[16/2^3 = 2\] virions. This suggests that the true assignment mechanism gives each daughter close to 50% of the mother cell’s virions, plus a small margin of error. Such a distribution may be achieved as a side effect of the distribution of LRV1 virions in parasites. Immunofluorescence imaging of viral capsid protein in Chapter 2 shows points of capsid distributed relatively uniformly across the parasite cytoplasm. Dividing the parasites in half would thus assign each daughter cell approximately 50% of the virions.

**5.8– Testing LRV1 Treatment in a Mouse Model of Leishmaniasis is Feasible**

In Appendix B, I present preliminary data and theoretical calculations supporting the feasibility of an attempt to inhibit or eliminate LRV1 from parasites in a mouse model of leishmaniasis. This work was motivated in part by a recent report that 7d2CMA is capable of inhibiting Zika virus in a mouse model. (Zmurko et al. 2016) The EC50 of 7d2CMA against Zika in cell culture (10 µM) was similar to that for LRV1 in cell culture (5 µM). The other motivating factor was the discovery that 1 µM 2CMA, a concentration previously thought completely ineffective, could slowly eliminate LRV1 from infected parasites in culture.

The pattern of virus loss under these conditions suggests that rather than slowly diluting the virus out of all cells at the same time, low concentrations of 2CMA seemingly increase the chances of stochastic virus loss during cell division. Interestingly, this result sheds some light on the validity of an assumption made in implementing my simulation of LRV1 and parasite replication. When simulating the distribution of virions to daughter cells upon parasite division, I found that the probability of randomly assigning all the virions to one daughter had a strong influence on the simulation (discussed in greater detail in section 5.7). Too large of a probability generated a growing population of parasites cured by random loss upon cell division. The resulting population distribution, where most parasites contain 16 virions and a growing minority
contains none, closely resembles the long-term effect of 1 µM 2CMA treatment. Given this similarity, I suspect that 1 µM 2CMA has a small effect on the rate of LRV1 replication that, although it does not prevent most parasites from recovering their full population of 16 virions prior to cell division, does slow replication sufficiently that a minority of parasites divide before the virus population has recovered. In such parasites, the probability of stochastically assigning the virions to one daughter cell rises significantly.

Based on these encouraging results, I utilized pharmacokinetic data collected for Hepatitis C Virus research to predict the behavior of 2CMA and 7d2CMA in mice. (Eldrup et al. 2004; Olsen et al. 2004) After comparing the available dosing routes for mice, I determined that the optimal dosing regimen is daily subcutaneous injections of 80 mg/kg 7d2CMA. This scheme is relatively simple to perform, relatively low stress for the mice, and should deliver the greatest amount of 7d2CMA to the parasites. Deciding when to begin 7d2CMA treatment will depend on the precise question being asked. The most fundamental question is whether any drug could reduce the effects of LRV1 on disease severity by eliminating or inhibiting the virus. For this purpose, we want to use the highest dose possible as soon as the mice are infected. This yields the highest probability of seeing any effect on LRV1 or disease severity. The other major question is whether, in clinical leishmaniasis cases, inhibiting LRV1 would have any effect. In this situation, the patient would only receive treatment after they noticed a lesion developing. We know that cultured macrophages begin responding to LRV1 within hours of being infected with LRV1+ parasites (Ives et al. 2011), so it is possible that the deleterious immune response would be irreversibly induced before the patient went to the clinic. To simulate this situation, we can infect mice with LRV1+ parasites and wait to begin 7d2CMA treatment until lesions begin forming.
5.9– Tools for Improving Nucleoside Analog Phosphorylation in vivo

In Appendix B, I described a drug screen intended to identify compounds that could inhibit *Leishmania*, LRV1, or the newly-discovered *Leishbunyavirus* (LBV). When we first designed this screen, we had three goals in mind. First, we were unsure if 7d2CMA was potent enough to use against LRV1 in mice, so we hoped to improve it or another compound’s potency. Second, with the recent discovery of LBV and its impact on disease severity, we wanted a screen that would identify LBV inhibitors in addition to *Leishmania* and LRV1 inhibitors. Third, we hypothesized that compounds artificially phosphorylated by the thymidine kinase (TK) could represent potential candidates for phosphoramidate pro-drugs that would avoid the limitations of the initial nucleoside phosphorylation step. (McGuigan et al. 2010) Such pro-drugs could also escape the promiscuous nucleoside hydrolases that form an integral part of *Leishmania* nucleoside metabolism. (Carter et al. 2008)

Based on the limited panel of compounds I tested, this approach did not yield any improvements or potential new nucleoside starting points for addressing the problems it was designed to solve. Although several compounds appeared to become moderate LRV1 inhibitors in the TK-expressing parasites, none are strong candidates for further study. There are a number of potential explanations for the dearth of solid hits. The compounds screened may not actually be good substrates for one of the activating enzymes or for the RNA polymerase itself. Treatment with immucillins may not inhibit the parasite nucleoside hydrolases sufficiently to avoid degrading the compounds. For LBV, however, we have insufficient data to draw solid conclusions. It may be worthwhile to screen a number of additional compounds for LBV inhibition to get a better idea of the screen’s effectiveness for that virus.
5.10– Conclusions

This work covers the discovery and characterization of a potential lead compound for preventing LRV1-associated disease exacerbation in humans (Chapter 3). In the process, it also provides an excellent example of the importance of the *Leishmania* purine salvage pathway in designing drugs for the parasite. Although the salvage pathway avidly accumulates 2CMA-TP and enables its potency in parasites (Chapter 4), the critical role of nucleoside hydrolases within the pathway also appears to prevent the activation of a number of compounds which would otherwise inhibit LRV1 (Appendix B). Beyond identifying a promising lead compound for future development, the information contained herein also provides the tools needed to efficiently screen for compounds inhibiting *Leishmania*, LRV1, and LBV.
5.11 – References


Appendix A: Exploration of genetic and pharmacological manipulations to improve the sensitivity of *Leishmania guyanensis* to nucleoside analogs
Preface

SMB and JIR designed the experiments. JIR performed the experiments and analyzed the data with technical assistance from Katherine Owens, George Lye, Natalia Akopyants and Erin Brettmann. Immucillins were the kind gift of Dr. Vern Schramm. The first draft of this appendix was written by JIR and the final version presented here incorporates comments from SMB.
A.1–Introduction

*Leishmania* parasites utilize a complex network of enzymes to scavenge nucleosides and nucleobases from the extracellular milieu and interconvert them as needed. Purines, in particular, are avidly scavenged because the parasites lack a *de novo* synthesis pathway. (Carter et al. 2008) The nucleoside salvage pathways are thus logical targets for anti-*Leishmania* drugs, but also provide a robust avenue to inhibit *Leishmania* viruses, as seen in chapters 3 and 4. Because the *Leishmania* RNA virus (LRV) polymerase is only inhibited by nucleoside triphosphates, this limits one to analogs that are efficiently converted to nucleoside triphosphates by the parasite. Another constraint is that nucleosides in *Leishmania* can be hydrolyzed to nucleobases by a promiscuous nucleoside hydrolase (NH) enzyme. (Carter et al. 2008; Cui et al. 2001; Shi et al. 1999) For nucleosides other than adenosine, for which a dedicated adenosine kinase has been described, this may be the primary route of nucleoside metabolism. (Datta et al. 1987; Bhaumik and Datta 1988) For nucleoside analogs with modifications on the ribose moiety, hydrolysis by this NH effectively degrades the compound by separating the otherwise normal nucleobase from the modified ribose. An additional limitation is that the initial activation step from nucleoside to nucleoside monophosphate step is rate-limiting in many organisms. (McGuigan et al. 2010) Circumventing these restrictions could allow identification and development of more potent parasite or LRV1 inhibitors.

This concept has already been implemented for other antiviral nucleosides. For example, the new phosphoramide pro-drugs against Hepatitis C Virus are nucleoside monophosphate analogs that bypass the relatively slow initial phosphorylation step. (Kirby et al. 2015; McGuigan et al. 2010) Another tactic used in gene therapy research has been the development of enzymes which promiscuously activate existing nucleoside analogs. The best example of this is the Herpes
Simplex Virus (HSV) thymidine kinase (TK), which has been engineered to increase its affinity for other nucleoside analogs such as ganciclovir. (Black et al. 2001) Ganciclovir is not normally phosphorylated well by human cells, so introduction of this mutant TK gene dramatically increases their susceptibility to the drug. Originally, this gene was envisioned as a potential method of gene therapy, whereby relevant cells would be made to express the promiscuous TK, allowing their selective elimination with ganciclovir. It has also been employed in Leishmania major as a negative selectable marker for genetic screens. (LeBowitz et al. 1992; Davoudi et al. 2005; Muyombwe et al. 1997) In a similar vein, expressing this TK in Leishmania could increase or enable activation of nucleoside analogs and thus reduce the dose required to eliminate LRV.

However, this TK gene would still be competing with Leishmania NH enzymes for nucleoside analogs. Dr. Vern Schramm’s research group has developed a class of nucleoside analogs that could inhibit this competition. These compounds, called immucillins, were originally designed by the Schramm lab to inhibit human purine nucleoside phosphorylase, an enzyme not found in Leishmania that also hydrolyzes nucleosides. (Ho et al. 2010; Miles et al. 1998) The defining characteristic of immucillins is the replacement of the ether group of the ribose sugar with a secondary amine (Figure A-1). Later work by the Schramm lab found that some immucillins inhibit the activity of the promiscuous Leishmania NH in vitro and the growth of certain species of parasites in vivo. (Freitas et al. 2015a; Shi et al. 1999; Freitas et al. 2015b) Treating parasites with immucillins, either alone or in a TK-expressing line, could reduce nucleoside analog degradation and improve potency.

In selecting a Leishmania strain to test the TK and immucillin combination, we chose to deviate from our previous work, which largely utilized L. guyanensis MHOM/BR/75/M4147 (LgyM4147). Instead, we utilized L. guyanensis LEM1684 (LgyLEM1684, WHO code
MHOM/BR/88/IM-3471), provided by U. Montpellier, France, which also bears LRV1. Additionally, LgyLEM1684 is also infected by a new *Leishmania* virus. This bunyavirus-like species, called *Leishbunyavirus* (LBV), was recently discovered by our lab. (Akopyants et al. 2016) Most *Leishmania* strains infected with LBV contain species LBV2. LBVs, like other bunyaviruses, are tri-segmented negative-sense ssRNA viruses. Unpublished work by our lab has shown that LBV2 is associated with considerable pathology in our mouse model of leishmaniasis. Screening of a large number of *Leishmania* isolates from South America has shown that LBV2 is very common (unpublished). Both of these factors suggest that LBV2 may prove to be even more important than LRV1 for determining the severity of human leishmaniasis. Working with LgyLEM1684 instead of LgyM4147 therefore provides an opportunity to screen for inhibitors of *L. guyanensis* and its two clinically relevant viruses at the same time.

**A.2– Methods**

**A.2.1 – Parasite Strains and Media**

This work was performed with two parasite strains: *L. guyanensis* LEM1684 (Lgy1684) and *L. major* MHOM/SU/73/5-ASKH (Lmj5ASKH; from Dr. Joaquim Clos, Hanover, Germany) Lg1684 was cultured in Schneider’s medium (Sigma), prepared as in Chapter 3. (Kuhlmann et al. 2017) Lmj5ASKH was grown in M199 medium plus 40 mM HEPES (pH 7.4), 0.1 mM adenine, 1 μg/mL biotin, 5 μg/mL hemin, 50 U/mL penicillin, 50 μg/mL streptomycin, 10% heat-inactivated fetal bovine serum, and 2 μg/mL biopterin. Culture density was measured using a Coulter counter (Beckman Coulter).
A.2.2 – Creation of TK-Expressing Parasites

Thymidine kinase mutant SR39 was amplified with PCR primers (Table A-1) that added a 5’ consensus CCACC Kozak sequence; a 3’ stop codon; and flanking AflIII and BglII restriction sites to allow inserting the gene into pIR-series vectors. This PCR product was then blunt-end ligated into pCR-Blunt (Invitrogen) and sequenced using the amplification primers and two internal primers (Table A-1). The TK gene was excised with AflIII and inserted into the (a) site of pIR3-HYG-LUC(b) (B7096), generating pIR3-HYG-mSR39TK(a)-LUC(b) (B7623) (Figure A-2). The TK construct was linearized with SwaI and transfected into LgyLEM1684 and Lmj5ASKH using the high voltage protocol. (Robinson and Beverley 2003) Transfected clones were selected on semi-solid medium containing 200 μg/mL or 50 μg/mL hygromycin for LgyLEM1684 and Lmj5ASKH, respectively. Four clones from each strain were grown up in liquid medium under the same hygromycin selection. For future work, clones LgyLEM1684 #12 and Lmj5ASKH #10 were selected because they exhibited the greatest sensitivity to ganciclovir (Figure A-4).

A.2.3 – Measuring TK Activity via Ganciclovir Sensitization

Active thymidine kinase was detected using its ability to activate ganciclovir, thereby inhibiting parasite growth. The parental strains of LgyLEM1684 and Lmj5ASKH were grown in the presence of 0, 1, 10, or 100 μg/mL ganciclovir for 48 hours and the culture densities were measured. Clones transfected with the TK construct were grown with or without 10 μg/mL ganciclovir until the untreated cultures reached a density of approximately 1×10^7 cells/mL. Growth rates were calculated by fitting the culture density data to an exponential growth equation.
A.2.4 – Measuring LRV1 and LBV Levels in Parasites

LRV1 levels were measured using a standard protocol previously developed in the lab, described in more detail in chapters 2 and 3. In brief, 2×10^7 parasites were fixed with 2% paraformaldehyde, stained with anti-capsid antibody, and visualized with an AlexaFluor-488 linked secondary antibody. Capsid staining levels were then measured in single cells using flow cytometry.(Kuhlmann et al. 2017)

LBV levels were measured using qRT-PCR. Total parasite RNA was extracted from 1×10^8 parasites using 350 μL TRIzol reagent and the Direct-Zol RNA miniprep kit (Zymo Research), according to the manufacturer’s instructions. DNA was digested with DNase I (Ambion) for 1 hour at 37°C followed by cleanup with an RCC-25 column purification kit (Zymo Research). cDNA was synthesized using the SuperScript III kit and random hexamer primers (Invitrogen). Negative controls without reverse transcriptase were also performed. qPCR reactions were set up using Power SYBR Green master mix (Applied Biosystems) and run on a ViiA 7 thermocycler (Applied Biosystems). Relative LBV levels were measured using primers generating a 103-bp product from the middle of the LBV RNA polymerase gene (Table A-2, pair 1). As an internal control, levels of RNA from the housekeeping gene KMP11 were measured using primers generating a 100-bp product (Table A-2). These reactions were run in triplicate and negative controls were run in duplicate. To estimate primer efficiency, 1 μL of cDNA from each reaction was pooled and diluted 1:5, 1:25, and 1:125. Each dilution was amplified in duplicate using the LBV or KMP11 primers. The PCR protocol included initial denaturation for 10 min. at 95°C followed by 40 cycles of denaturation for 15 sec. at 95°C and amplification for 1 min. at 60°C. Ct values were calculated using the ViiA 7 software. Relative quantitation was
accomplished via the Pfaffl method, which uses 1:5 serial dilutions of mixed cDNA to correct for non-ideal primer efficiencies. (Pfaffl 2001)

A.2.5 – Parasite Susceptibility to Immucillins

We received Immucillin A (IA), 4-deaza-1-aza-2-deoxy-1-(9-methylene)-Immucillin A (DADMe-IA or DIA), Immucillin H (IH), and DADMe-Immucillin H (DIH) as kind gifts from Dr. Vern Schramm (Figure A-1). Wild-type Lgy1684 parasites were seeded at 2×10^5 cells/mL in 5 mL of Schneider’s medium plus 100 µM immucillin. Parasite growth was monitored for 48 hours, after which LRV1 levels were measured using the flow cytometry technique described in the previous section.

A.2.6 – Screening for Efficacy Enhancement by TK or Immucillins

The compounds used in this screen (Table A-3) were initially obtained for use in the LRV1 inhibitor screen described in Chapter 3 (see Table S1). All compounds were tested at 100 µM from 50 mM stocks in DMSO. For the first round of screening, compounds were tested against the parental Lgy1684 strain or Lg1684 TK clone #12 (Lgy1684-TK, see Figure A-4A). An additional parental Lgy1684 culture was treated with drug plus 5 µM of a mixture of all four immucillins, followed with another dose in 24 hours. Parasite growth was monitored for 48 hours before cells were harvested for measuring LRV1 and LBV levels. To reduce the number of samples in subsequent rounds of screening, compounds were tested against Lg1684 or Lg1684-TK treated with the immucillin cocktail.

A.3 – Results

A.3.1 – Parasites Express Active TK Enzyme

In order to potentially improve the phosphorylation of nucleoside analogs, we over-expressed the promiscuous HSV TK mutant SR39 in Lgy1684 and Lmj5ASKH. I confirmed that
up to 100 μg/mL ganciclovir has no effect on Lmj5ASKH and Lgy1684 parasites (Figure A-3). By contrast, clones of Lmj5ASKH and Lgy1684 containing the TK and luciferase genes were strongly inhibited by 10 μg/mL ganciclovir (Figure A-4). This demonstrated that those clones expressed significant amounts of active TK. Lg1684 TK clone #12 (Lg1684-TK) was utilized for subsequent experiments since it exhibited the strongest growth inhibition.

A.3.2 – Lg1684 and its LRV1 Strain are Insensitive to Immucillins

Based on prior reports, we expected that immucillins would inhibit parasite growth. (Freitas et al. 2015a; Freitas et al. 2015b) Since immucillins are nucleoside analogs, it was also possible that they would inhibit LRV1 as well. To test this, I grew Lgy1684 parasites in the presence of 100 μM of each immucillin for 48 hours. Growth rate and LRV1 levels per cell were then measured (Figure A-5). None of the compounds inhibited parasite growth, suggesting that, unlike L. infantum and L. amazonensis, L. guyanensis is not susceptible to these compounds. In addition, LRV1 was not inhibited by any immucillin.

A.3.3 – LRV1 Screen Identifies Two Compounds Possibly Activated by TK

To test the effects of immucillins and TK on compound efficacy, I began by screening compounds similar to known LRV1 inhibitors 2CMA and 7d2CMA. I began by testing 2’-C-methyl-nucleosides (Figure A-6) against Lgy1684, Lgy1684-TK, and Lgy1684 treated with immucillins. Based on previously published data, I utilized two 5 μM doses of immucillin cocktail, one added at the start of the experiment and the second after 24 hours. The Schramm group observed that this dosing scheme significantly improved the potency of immucillins IA and IH against L. infantum. (Freitas et al. 2015b) As expected, 2CMA and 7d2CMA strongly inhibited LRV1 levels. Parasite growth was inhibited somewhat more for Lgy1684 than in our original screen with LgyM4147, but this may be explained by differences between strains.
Treating Lgy1684 parasites with both the immucillin cocktail and the compounds did not induce additional inhibition of parasite growth or LRV1 levels. The TK gene, however, induced a 25% reduction in LRV1 capsid levels in parasites treated with 2’-C-methyl-cytosine (2CMC) without changing parasite growth. Since 2CMC is not normally active against LRV1 in parasites, this suggests that phosphorylation by TK might have allowed 2CMC to be activated and inhibit LRV1. However, this is only one replicate and a relatively small improvement, so more work would be required to confirm it.

In a second, larger round of screening, I combined the TK and immucillin treatments to identify compounds of interest more efficiently. A series of 2’-fluoro-2’-deoxy- (E1-E5) and cyclobutyl-adenine nucleoside analogs (E8-E10) were tested using the same protocol as for the initial screen. None of the compounds caused more than 20% reduction in parasite growth rates (Figure A-7A). The cyclobutylyl-adenine analogs, however, appeared to reduce LRV1 levels (Figure A-7B). In wild-type parasites, they induced ~20% increases in LRV1 levels, consistent with the results from the initial screen in Chapter 3 (see Table S2). In Lgy1684-TK parasites treated with immucillins, they induced 20-40% reductions in LRV1 levels relative to untreated controls. This data suggests that cyclobutyl-adenine derivatives, in particular 3’-hydroxyethyl cyclobutyl-adenine, might be able to inhibit LRV1 once activated. As with 2CMC, the effects reported here represent a single experiment and are relatively small, so additional experiments would be required to make firm conclusions.

A.3.4– Preliminary LBV screen yields no hits

Previous work in our lab (Dr. F. Matthew Kuhlmann, unpublished) showed that none of the compounds originally tested against LRV1 could inhibit LBV. To test whether phosphorylation by TK could allow otherwise inactive compounds to inhibit LBV, I measured
virus levels in wild-type Lgy1684 or Lgy1684-TK after treating with 2CMC or 2CMG. These compounds are similar to 2CMA, but the arrangement of the purine and pyrimidine salvage pathways in *Leishmania* suggests that in wild-type parasites they would be destroyed by nucleoside hydrolases and not phosphorylated. (Carter et al. 2008) Furthermore, the fact that 2CMC inhibited LRV1 somewhat in Lg1684-TK suggested that 2CMC might have been activated by the TK. RNA purified from these parasites was analyzed by qRT-PCR using primers amplifying an internal portion of the virus’ RNA polymerase gene. As Dr. Kuhlmann previously observed, neither compound changed the amount of LBV in wild-type parasites. The TK gene also had no significant effect on LBV levels (Figure A-8).

**A.4 – Discussion**

In this preliminary study, I report the tentative identification of multiple nucleoside analogs that may inhibit LRV1 when phosphorylated by a promiscuous TK enzyme. Although the compounds identified in this work are not particularly potent, they illustrate the utility of a promiscuous TK gene to locate lead compounds worthy of future study. Obtaining tentative hits from two structurally unrelated classes of nucleoside analog in a screen of 12 compounds suggests that more potent LRV1 inhibitors could easily be found through further screens.

With the goal of enhancing phosphorylation of nucleoside analogs in *Leishmania*, I expressed a promiscuous thymidine kinase in Lgy1684. Using ganciclovir susceptibility as a proxy for TK activity, I showed that the TK-expressing parasites produced significant amounts of active TK (Figure A-4). In a parallel effort to block drug degradation by the *Leishmania* nucleoside hydrolases, I treated parasites with a cocktail of four immucillins. Individually, none of these compounds had any effect on Lg1684 growth or LRV1 levels (Figure A-5). This is not entirely surprising, since these compounds have not been tested against members of the *Viannia*
sub-genus and thus might not be active against them. Furthermore, although all available immucillins were utilized to maximize potential NH inhibition, not all of them have been shown to inhibit *Leishmania* or NH. Work published by the Schramm lab showed that IA and IH inhibit both parasites and NH, while DIH inhibits parasites but not NH and DIA inhibited neither. (Freitas et al. 2015b)

Screening for changes in anti-LRV1 activity induced by TK or immucillins identified two distinct types of compound. The first, 2CMC, induced a 20% drop in LRV1 levels when tested in TK-expressing parasites (Figure A-6B). This compound is the cytidine version of 2’-C-methyl-adenosine, the first anti-LRV1 compound we identified, so its activity against LRV1, if confirmed, would not be surprising. *Leishmania* parasites are thought to incorporate cytidine into the pyrimidine nucleotide pool by first hydrolyzing it to cytosine. (Carter et al. 2008) This step would degrade 2CMC in wild-type parasites by removing the methyl-ribose from the cytosine nucleobase.

The second type of compound tentatively identified in this screen was a group of structurally-similar cyclobutyl-adenine compounds. In wild-type parasites, these compounds increased the levels of LRV1. In Lg1684-TK treated with immucillins, they reduced LRV1 levels by 25-35% (Figure A-7B). Compound E10, 3’-hydroxyethyl-cyclobutyl-adenine, was the most affected, going from a 25% increase in wild-type parasites to a 35% decrease with TK and immucillins. The fact that all three examples of this compound class in the screen may be activated to varying extents suggests that those results should be followed up and other related compounds should be explored further for more potent LRV1 inhibitors.

The Lgy1684 parasite strain was selected for this work because it also contains the LBV virus, a novel bunyavirus-like negative-sense ssRNA virus recently discovered by our lab. This
allows screening for *Leishmania*, LRV1, and LBV inhibitors at the same time. A preliminary look at the effect of TK activation on LBV levels did not reveal any significant effects. Since 2CMC was activated sufficiently to slightly inhibit LRV1 in the same experiment, this result suggests that LBV might be resistant to 2CMC and its triphosphate.

This screening technique could also be extended to search for inhibitors of other, less well-understood *Leishmania* viruses. When the LRV1 inhibitor 2’-C-methyl-adenosine (2CMA) was identified (Chapter 3), we tested it against LRV2, an Old World LRV species infecting *L. major* 5ASKH, and found it had no effect. Using the 2CMA-TP assay I developed for LRV1, I also found that *L. major* 5ASKH concentrates 2CMA-TP approximately as much as *L. gy.* M4147. This implies that LRV2 itself is resistant to 2CMA-TP. I have expressed active TK in *L. major* 5ASKH, so I could extend my pro-drug screen to find LRV2 inhibitors. Another potential target is Bottom virus, another *Leishmania* virus we have recently uncovered. However, we know little about this virus, so screening for inhibitors might be premature.
A.5–References


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### Table A-1: Primers used to amplify and sequence TK.

**Primers used to amplify and sequence TK.**

(mSR39-TK) This primer pair is for amplifying TK mutant SR39. It includes flanking AflII and BglII restriction sites for insertion into pIR-series vectors. **(Sequencing)** These internal primers allow sequencing the ends of the TK ORF.

<table>
<thead>
<tr>
<th>Primer Pair</th>
<th>ID #</th>
<th>Direction</th>
<th>Sequence (5'→3')</th>
</tr>
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<tbody>
<tr>
<td>mSR39-TK</td>
<td>B7075</td>
<td>Forward</td>
<td>CCGGGCTTAAAGATCTCCACCATGGCTTCGTACCCCGG</td>
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<tr>
<td></td>
<td>B7076</td>
<td>Reverse</td>
<td>CCGGGCTTAAAGATCTTCTAGTACCTCCCCCATCTCCGG</td>
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<tr>
<td>Sequencing</td>
<td>B2288</td>
<td>Reverse</td>
<td>GCAGTAGCGTGCGCATTPTT</td>
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<tr>
<td></td>
<td>B2289</td>
<td>Forward</td>
<td>CCCCACGGCGGACCTGT</td>
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</table>
Table A-2: Primer pairs for quantifying LBV virus levels in L. guyanensis LEM1684.

The LBV primers amplify ~100 bp segments from the middle of the largest viral genome segment. Pair 1 has superior performance over Pair 2. The KMP11 primers amplify a ~100 bp segment of the KMP11 housekeeping gene. (Zangger et al. 2013)

<table>
<thead>
<tr>
<th>Primer Pair</th>
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<th>Sequence (5’→3’)</th>
</tr>
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<tbody>
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<td>GGTACAAGGGAGGGTCTTTATG</td>
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<td></td>
<td>B7938</td>
<td>Reverse</td>
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<tr>
<td>LBV Pair 2</td>
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<td>GAGGAGCCTCGGAATAAGAATG</td>
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<tr>
<td></td>
<td>B7940</td>
<td>Reverse</td>
<td>TACGGAGGTTTATTGGA</td>
</tr>
<tr>
<td>KMP11 Standard</td>
<td>B5548</td>
<td>Forward</td>
<td>GCCTGGATGAGGAGTTCAACA</td>
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<tr>
<td></td>
<td>B5549</td>
<td>Reverse</td>
<td>GTGCTCCTTCATCTCGGG</td>
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Table A-3: Compounds tested in this study.

More details about each compound are available in Chapter 3, Table S1.

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</tr>
<tr>
<td>E2</td>
<td>2'-fluoro-2'-deoxyuridine</td>
</tr>
<tr>
<td>E3</td>
<td>2'-fluoro-2'-deoxyadenosine</td>
</tr>
<tr>
<td>E4</td>
<td>2'-fluoro-2'-deoxyguanosine</td>
</tr>
<tr>
<td>E5</td>
<td>2'-fluoro-2'-deoxyinosine</td>
</tr>
<tr>
<td>E6, 2CMA</td>
<td>2'-C-methyl-adenosine</td>
</tr>
<tr>
<td>E7, 2CMG</td>
<td>2'-C-methyl-guanosine</td>
</tr>
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<td>2CMC</td>
<td>2'-C-methyl-cytidine</td>
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<td>7d2CMA</td>
<td>7-deaza-2'-C-methyl-adenosine</td>
</tr>
<tr>
<td>E8</td>
<td>3'-azido-3'-hydroxyethyl α-cyclobutyl-adenine</td>
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<tr>
<td>E9</td>
<td>3'-azido-3'-hydroxyethyl β-cyclobutyl-adenine</td>
</tr>
<tr>
<td>E10</td>
<td>3'- hydroxyethyl cyclobutyl adenine</td>
</tr>
</tbody>
</table>
A.7 – Figures

Immucillin A

DADMe-Imm. A

Immucillin H

DADMe-Imm. H

Figure A-1: Chemical structures of the immucillins used in this study.
Figure A-2: Diagram of Leishmania construct expressing the HSV TK gene.

TK gene is marked by a blue arrow (mSR39-TK). The construct (SMB7623) is based on pIR3-HYG, which includes intergenic regions (IR) and splice acceptor sites (SA) that allow proper maturation of mRNAs transcribed from 3 positions in the construct. The first contains the TK gene, the second contains a luciferase gene for visualizing parasites in mice, and the third contains the hygromycin resistance gene (HYG). When linearized with Swal, it can recombine into the ribosomal small subunit (SSU) locus using the L. major SSU flanking sequences.
Figure A-3: Effect of ganciclovir on growth of wild-type Leishmania parasites.

Cultures were grown for 48 hours in the presence of varying amounts of ganciclovir. No growth inhibition was observed.
A. Figure A-4: Effect of 10 μg/mL ganciclovir on parasites transfected with TK expression construct.

B. Growth rates of LgyLEM1684 (A) or Lmj5ASKH (B) TK-transfected clones grown for 72 or 96 hours, respectively, in the presence of 10 μg/mL ganciclovir (black bars) or no drug (white bars).
Figure A-5: LgyLEM1684 and its LRV1 strain are insensitive to a panel of immucillins.

(A) Growth rates of cultures grown with 100 µM of each compound. (B) LRV1 levels after growth for 48 hours in each compound, as measured by capsid staining intensity via flow cytometry. (n=1)
Figure A-6: Initial round of TK/immucillin screening.

LgyLEM1684 parasites were grown in the presence of 100 µM of each compound. (A) Effect of each compound on the growth rate of the parasites. (B) Effect of each compound on the levels of LRV1 capsid staining in parasites. (n=1)
Figure A-7: Second round of screening with TK/immucillins.

Graphs compare drug effects in wild-type Lg1684 (black bars) or Lg1684-TK treated with the immucillin cocktail (white bars). Parasites were grown in the presence of 100 µM of each compound. (A) Effect of each compound on the growth rate of the parasites. (B) Effect of each compound on the levels of LRV1 capsid staining in parasites. (n=1)
Figure A-8: Levels of LBV virus RNA are unchanged by 2CMC and 2CMG, even in the presence of the TK enzyme.

RNA from compound-treated parasites was analyzed by qRT-PCR (n=3), using KMP11 transcript levels as an internal control (n=3).
Appendix B: Feasibility of LRV1 Cure in Mouse Model of Leishmaniasis
Preface

SMB and JIR designed the experiments. JIR performed the experiments and analyzed the data. JIR performed the background research and modeling with technical advice from Suzanne Hickerson. The first draft of this appendix was written by JIR and the final version presented here incorporates comments from SMB.
Leishmania RNA Virus 1 (LRV1) has been shown to play a role in disease severity in both mouse models of leishmaniasis and human Leishmania infections. (Periera et al. 2013; Castiglioni et al. 2017; Hartley et al. 2016; Ives et al. 2011; Bourreau et al. 2015; Cantanhede et al. 2015; Ito et al. 2015) It is possible that this increase in disease severity could be avoided by reducing or eliminating LRV1 from parasites in a mammalian host. The potential merit of this concept was reinforced by work showing that immunizing mice with the LRV1 capsid protected them from virus-enhanced disease severity. (Castiglioni et al. 2017) However, macrophages infected with LRV1+ parasites produce increased pro-inflammatory cytokines within 6 hours of infection. (Ives et al. 2011) This suggests that the immune response to LRV1 may occur soon after infection. Thus, treating LRV1 only after a lesion develops might be too late to have any effect on disease progression. To answer this question, we need to be able to inhibit LRV1 in a mouse model of Leishmania infection.

In Chapter 3, we describe the discovery of two nucleoside analogs, 2’-C-methyladenosine (2CMA) and 7-deaza-2CMA (7d2CMA), which inhibit LRV1 in cultured parasites. These compounds were both developed initially as Hepatitis C Virus (HCV) inhibitors. (Eldrup et al. 2004; Olsen et al. 2004) Based on its rapid degradation in animals, 2CMA was not pursued as an HCV inhibitor. (Eldrup et al. 2004) 7d2CMA, on the other hand, was not degraded by human enzymes and was able to inhibit HCV in a chimpanzee model. (Eldrup et al. 2004; Carroll et al. 2009) Recently, 7d2CMA was also shown to inhibit Zika virus in cell culture with an EC50 similar to the one we measured against LRV1. The drug was then successfully utilized to reduce Zika virus replication in a mouse model of infection. (Zmurko et al. 2016) Based on these results, we hypothesized that 7d2CMA might be sufficiently potent to significantly reduce or
eliminate LRV1 in our mouse model of leishmaniasis. Furthermore, inhibiting LRV1 would reduce its deleterious effects on the mouse immune response.

**B.2 – Methods**

**B.2.1 – Treatment of LRV+ Parasites with Low 2CMA**

*L. guyanensis* M4147 (LgM4147) parasites identical to those used to originally identify 2CMA were grown for this experiment using the same formulation of Schneider’s medium (see Chapter 3 for more details). (Kuhlmann et al. 2017) Log phase cells were passed into 5-mL cultures of Schneider’s medium at 2×10⁵ cells/mL and grown in the presence of 1 µM 2CMA or DMSO for 48 hours. The culture density was measured using a Coulter Counter and cells were passed into fresh medium at 2×10⁵ cells/mL with 1 µM 2CMA or DMSO. At each passage, LRV1 levels were measured by flow cytometry using the protocol described in Chapter 2. Briefly, 2×10⁷ cells were fixed with 2% paraformaldehyde and stained with 1:20,000 anti-LRV1 capsid antibodies followed by 1:1000 AlexaFluor488-labeled goat anti-rabbit IgG. Parasites stained only with secondary antibody were used to measure background staining. DMSO-treated parasites were used as a positive control for LRV1+ parasites. LRV-low parasites were identified by gating on the range covered by background staining, while LRV-high parasites were those with capsid staining above background (Figure B-1).

**B.2.2 – Predicting the Pharmacokinetics of 2CMA and 7d2CMA in Mice**

The relevant pharmacokinetic (PK) parameters were taken from Olsen *et al.* where 7d2CMA was studied as a potential treatment for Hepatitis C virus (HCV). (Olsen et al. 2004) Parameters for 2CMA were obtained from Eldrup *et al.* where it was used as a baseline HCV inhibitor for comparison to other, more potent nucleosides. (Eldrup et al. 2004) Lacking measured parameters for mice, those measured for rats were used instead. Standard PK equations
were utilized to predict the behavior of 2CMA and 7d2CMA upon dosing in mice. (Kallen 2007)

For these calculations, I needed to estimate the notional “volume” of distribution ($V_D$) for a
mouse. This allows one to predict the initial plasma concentration resulting from a given
intravenous (IV) dose of drug. By definition,

$$V_D = \frac{A(0)}{D(0)}$$ (1)

where $A(0)$ is the amount of drug administered and $D(0)$ is the resulting initial plasma
concentration.

The rate at which a drug is excreted or degraded in a particular animal is expressed as

$$rate \ of \ elimination = -\frac{dA(t)}{dt} = Cl_p(t)D(t)$$ (2)

where $Cl_p(t)$ is the clearance function. In linear PK systems, where the elimination rate is
independent of drug concentration, the value of $Cl_p(t)$ is usually constant. For the purposes of
these calculations, I assumed this was the case. As a first-order approximation, I also assumed
that elimination of 2CMA and 7d2CMA is described by an exponential equation, which is
usually true for linear PK systems:

$$A(t) = A(0) e^{-kt}$$ (3)

Taking the derivative of equation (3) and combining it with equation (2) gives

$$\frac{dA(t)}{dt} = -k A(0) e^{-kt} = -Cl_p D(t)$$ (4)

Setting $t = 0$ reduces equation (4) to

$$k A(0) = Cl_p D(0)$$ (5)

Thus the rate constant for elimination of drug is

$$k = \frac{Cl_p D(0)}{A(0)}$$ (6)

Since $k$ is an exponential rate constant, it can be rewritten as
\[ k = \frac{\ln 2}{t_{1/2}} \]  

where \( t_{1/2} \) is the time required to reduce \( A(0) \) by half. Published PK data on 7d2CMA and 2CMA included the values of \( Cl_p \) and \( t_{1/2} \). Combining equations (6) and (7) and solving for \( D(0) \) allows it to be calculated for any drug dose \( A(0) \)

\[ D(0) = \frac{A(0) \ln 2}{Cl_p \ t_{1/2}} \]  

(8)

Note that this also allows one to calculate the volume of distribution for the system, using equation (1)

\[ V_D = \frac{A(0)}{D(0)} = \frac{Cl_p \ t_{1/2}}{\ln 2} \]  

(9)

If \( V_D \) is assumed to be constant over time, the concentration of drug at time \( t \) becomes

\[ D(t) = D(0)e^{-kt} = \frac{A(0) \ln 2}{Cl_p \ t_{1/2}} \exp \left( -\frac{\ln 2}{t_{1/2}} t \right) \]  

(10)

Equation (10) was used to predict the plasma concentrations of 2CMA and 7d2CMA after an IV dose, where all drug is delivered immediately to the bloodstream. For oral doses of drug, the amount of drug delivered to plasma is a certain percentage of the original dose. This is given as bioavailability \( (B) \) and was corrected using the equation

\[ A(0) = B \times A_{oral} (0) \]  

(11)

For continuous dosing schemes such as implantable pumps, the steady-state drug plasma concentration can be calculated by noting that the rate of elimination from equation (2) will be equal to the rate of drug input \( (R) \)

\[ -\frac{dA(ss)}{dt} = Cl_p D(ss) = R \]  

(12)

Thus, at steady state the plasma concentration will be
\[ D(ss) = \frac{R}{Cl_p} \] (13)

This equation is also applicable to regularly repeated bolus doses. At steady state, the average rate of drug input will be

\[ R = \frac{B \times MD}{DI} \] (14)

where \( MD \) is the amount of drug in the recurring maintenance dose and \( DI \) is the time between doses. Combining equations (13) and (14) gives the average steady state plasma concentration

\[ \bar{D}(ss) = \frac{B \times MD}{Cl_p \times DI} \] (15)

Importantly, this equation shows that a given steady state concentration may be achieved using low doses at short intervals or large doses at long intervals. However, increasing the dose interval makes the actual plasma concentration \( D(t) \) become increasingly erratic.

To estimate the relative intensity of drug treatment achieved using a given dose and delivery method, I calculated the area under the curve (AUC). The AUC is the integral of drug concentration over the course of the experiment, and is an approximate measure of the total drug exposure for a particular dosing scheme. For IV routes, AUC was simply the integral of equation (10) from 0 to 24 hours. When considering oral dosing routes, I assumed that the drug was absorbed at a constant rate until the time of maximum concentration was reached, at which point the concentration began to decay exponentially.

**B.3– Results**

*B.3.1 – Low 2CMA Levels Progressively Cure LRV1+ Parasites*

One significant concern when designing a dosing regimen is whether drug plasma concentrations below the EC50 will have any effect. Based on previously reported titrations (Chapter 3), 1 \( \mu \)M 2CMA has no measurable effect on mean LRV1 levels in parasites after 48
hours. (Kuhlmann et al. 2017) To determine the long-term effect of low 2CMA doses on LRV1 levels, I treated LRV1+ LgyM4147 parasites with 1 µM 2CMA over 4 passages and 215 hours. At each passage, I analyzed LRV1 levels using flow cytometry. This revealed that the proportion of LRV1-low parasites increased linearly as time treated with 2CMA increased (Figure B-2). Interestingly, this change manifested as a LRV-low peak on the capsid-staining histograms (Figure B-1), rather than a uniform reduction in the LRV1 levels of all parasites. This pattern suggests that low doses of 2CMA induce LRV1 loss in a small proportion of parasites as they divide.

B.3.2 – Mouse Treatment with 7d2CMA is Feasible

Given that 2CMA concentrations well below its EC50 had a significant impact on LRV1 levels in parasites, I set out to predict whether similar concentrations could be achieved in our mouse model of leishmaniasis. I used previously published PK parameters for rats, making the assumption that metabolism would be similar in mice. (Eldrup et al. 2004; Olsen et al. 2004) In order for a daily dose of 2CMA to yield an average plasma concentration greater than its EC50 (~3 µM), it would be necessary to use 250 mg/kg doses. Due to the limited solubility of 2CMA and 7d2CMA (~8 mg/mL) this delivering this much drug would require using volumes at the extreme upper end of recommended limits (Table B-2). Furthermore, the plasma concentration of 2CMA would drop below 1 µM within 2.5 hours. Thus, 2CMA cannot be used to inhibit LRV1 in mice. The PK parameters for 7d2CMA are much more favorable. A 50 mg/kg daily dose would be sufficient to maintain an average plasma concentration above its 5 µM EC50. The 7d2CMA plasma concentration would also remain above 1 µM for 9 hours. This suggests that an effective plasma concentration of 7d2CMA could be achieved in mice.
Due to factors such as dose volume limitations and incomplete drug absorption, the route of administration has a strong effect on the amount of drug delivered. I evaluated the possible dosing routes based on three criteria: suitability for repeat dosing, technical skill required, and amount of drug delivered (Table B-2). (Diehl et al. 2001; Turner et al. 2011) Gastric gavage – injecting drug directly into the stomach – was used successfully to treat Zika virus with 7d2CMA. (Zmurko et al. 2016) However, it suffers from low bioavailability and large amounts of 7d2CMA are taken up by the liver. (Olsen et al. 2004) Subcutaneous injection is simple and safe for long-term repeated treatment and delivers the full dose without bioavailability issues or liver degradation. Intravenous injection has similar PK behavior, but requires skillful surgical installation of a port for extended experiments. Intraperitoneal injection – directly into the body cavity – has frequent complications and is subject to first-pass degradation in the liver. Intradermal and intramuscular injections are not typically used in mice because the skin and muscles are too small to withstand most injections, especially repeated ones. (Diehl et al. 2001) An alternative method is delivering the drug continuously via an implantable pump. These are able to maintain stable plasma concentrations over extended periods. However, the low solubility of 7d2CMA limits the amount of drug that can be delivered. (DURECT Corporation 2017a, b) Based on their simplicity and relative safety, subcutaneous injection and gastric gavage are the two best dosing options for 7d2CMA.

I utilized the equations described in the methods section to compare the amounts of drug delivered using once-daily subcutaneous injection and gastric gavage (Table B-1). Ultimately, the amount of drug delivered by a particular dosing scheme can be summarized by the integral of drug concentration over time, which is referred to as the area under the curve (AUC). I calculated this value, plus the maximum drug concentrations in plasma and time with drug above 1 µM if
mice were given 7d2CMA once daily. Based on the previously published treatment of Zika with 7d2CMA (Zmurko et al. 2016) and the maximum recommended dose volumes, I compared 7d2CMA plasma concentrations for daily 50 and 80 mg/kg doses. As a reference point, theoretical values were calculated where the plasma 7d2CMA concentration was held constant. This roughly corresponds to the amount of drug encountered by parasites in cell culture treated with a given concentration of drug. Due to the relatively low bioavailability of 7d2CMA (51%), gastric gavage could only achieve an AUC value of 108 hr*µM, on par with a theoretically constant concentration of 5 µM 7d2CMA. Subcutaneous injection, on the other hand, gives a much higher AUC value of 190 hr*µM. This demonstrates that subcutaneous injection of 7d2CMA would deliver significantly more drug than gastric gavage.

**B.4 – Discussion**

This work suggests that it will be feasible to inhibit LRV1 in our mouse model of leishmaniasis using 7d2CMA. The fact that treating parasites with 2CMA concentrations well below its EC50 still lowers LRV1 levels implies that even relatively limited amounts of drug would still have a significant effect on LRV1 levels in our mouse model. Based on a simple model using published PK data, it is possible to generate plasma 7d2CMA concentrations in our mouse model of leishmaniasis sufficient to inhibit LRV1. Thus, we should be able to test whether reducing or eliminating LRV1 during a *Leishmania* infection will avoid the exacerbated disease severity usually generated by LRV1+ parasites.

The long-term low 2CMA experiment shows that a concentration of 2CMA low enough to have no significant effect after one passage in culture is still able to reduce LRV1 levels in exposed parasites. Interestingly, this treatment generates a population of LRV1-low parasites rather than uniformly reducing the number of LRV1 virions in all parasites (Figure B-1). This
pattern suggests that low doses of 2CMA lead to the stochastic loss of LRV1 in a subset of cells. This fits with the fact that this strain has, on average, 15 virions per cell. (Kuhlmann et al. 2017) If drug pressure lowers the average number of virions per cell slightly, this would increase the probability of a daughter cell receiving no virions upon cell division. These relatively rare events would then slowly increase the proportion of LRV1-negative parasites, which would only be noticeable after long-term treatment with drug.

Another piece of evidence supporting the feasibility of inhibiting LRV1 with 7d2CMA is that it has previously been used to treat Zika virus in mice. (Zmurko et al. 2016) In cell culture, 7d2CMA has similar EC50s against Zika (10 µM) and LRV1 (5 µM). Since giving daily 50 mg/kg doses of 7d2CMA to Zika-infected mice via gastric gavage significantly inhibited Zika virus, we can be reasonably confident that the same dose would inhibit LRV1 as well. Another important implication of the Zika experiment is that 7d2CMA can be effective at sites other than the liver. The concentration of 7d2CMA in the liver has been shown to far exceed the plasma concentration (Olsen et al. 2004), but Zika virus is not liver-specific like HCV and thus would not benefit from that distribution.

Since 7d2CMA treatment seemed likely to have an effect on LRV1 in our mouse model of leishmaniasis, I evaluated various potential administration routes and dosing amounts. Gastric gavage was considered in light of its successful use to treat Zika virus. However, this route suffers from several drawbacks, including low bioavailability and the potential for fatal adverse events when dosing the mice. Subcutaneous injections avoid both of these problems, while also avoiding the initial round of liver metabolism. This can easily be seen by considering the predicted effects of giving 7d2CMA by either route (Table B-1), which shows that the average plasma concentration and total AUC are roughly twice as high when 7d2CMA is given by
subcutaneous injection. In fact, the total AUC from an 80 mg/kg subcutaneous injection of 7d2CMA would be significantly greater than if the plasma concentration were maintained at 5 µM, the drug’s EC50.

These PK predictions should also be considered in light of the washout experiments reported in Chapter 4. They demonstrated that 2CMA-TP has a half-life of 4-8 hours in parasites after 2CMA has been removed from the medium. This means that drug taken up and activated by parasites in mice following the daily drug dose would be retained longer than the plasma concentrations would suggest. As a result, the effect of a given dose of 7d2CMA on LRV1 in parasites in a mouse is likely to be significantly higher than predicted. This provides further support for the viability of inhibiting LRV1 in mice.

Taken together, this evidence suggests that treating infected mice daily with 80 mg/kg 7d2CMA via subcutaneous injection is likely to inhibit LRV1. By starting 7d2CMA treatment either immediately or after a lesion has begun forming, we can determine whether delayed treatment of LRV1 would avoid the enhanced parasite numbers and pathology normally characteristic of LRV1+ parasites. If delayed inhibition of LRV1 reduces its effects in our mouse model, then further efforts should be undertaken to identify a drug capable of treating LRV1 in human leishmaniasis.
B.5 – References


Zmurko J, Marques RE, Schols D, Verbeken E, Kaptein SJ, Neyts J (2016) The Viral Polymerase Inhibitor 7-Deaza-2'-C-Methyladenosine Is a Potent Inhibitor of In Vitro
Zika Virus Replication and Delays Disease Progression in a Robust Mouse Infection Model. PLoS Negl Trop Dis 10 (5):e0004695. doi:10.1371/journal.pntd.0004695
Table B-1: Summary of once-daily dosing options for treating mice with 7d2CMA.

AUC is an approximate measure of the total intensity of the drug dose over a given amount of time (here 24 hours), and is calculated as the integral of drug concentration over that time. The AUC values allow one to sort the dosing routes by how much drug they deliver to the plasma. The “Theoretical” route represents an idealized dosing route where the plasma 7d2CMA concentration is held constant. This provides an approximation of the amount of drug seen by parasites in culture. For these, the AUC is simply the concentration times 24 hours.

<table>
<thead>
<tr>
<th>Administration Route</th>
<th>Dose</th>
<th>AUC (24 hrs) (hr*μM)</th>
<th>Peak [7d2CMA] (μM)</th>
<th>Time Above 1 μM [7d2CMA]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theoretical (low)</td>
<td>1 μM</td>
<td>24</td>
<td>1 μM</td>
<td>n/a</td>
</tr>
<tr>
<td>Gastric Gavage</td>
<td>50 mg/kg</td>
<td>67</td>
<td>26 μM</td>
<td>7.6 hrs</td>
</tr>
<tr>
<td>Gastric Gavage (high)</td>
<td>80 mg/kg</td>
<td>108</td>
<td>42 μM</td>
<td>8.6 hrs</td>
</tr>
<tr>
<td>Subcutaneous</td>
<td>50 mg/kg</td>
<td>119</td>
<td>52 μM</td>
<td>9.1 hrs</td>
</tr>
<tr>
<td>Theoretical (EC50)</td>
<td>5 μM</td>
<td>120</td>
<td>5 μM</td>
<td>n/a</td>
</tr>
<tr>
<td>Subcutaneous (high)</td>
<td>80 mg/kg</td>
<td>190</td>
<td>82 μM</td>
<td>10.2 hrs</td>
</tr>
<tr>
<td>Theoretical (high)</td>
<td>10 μM</td>
<td>240</td>
<td>10 μM</td>
<td>n/a</td>
</tr>
</tbody>
</table>
Table B-2: Summary of treatment options for administering 7d2CMA to lab mice.

Information obtained from Diehl et al. or Turner et al. unless otherwise noted. (Diehl et al. 2001; Turner et al. 2011)
### Gastric Gavage

<table>
<thead>
<tr>
<th>Pros</th>
<th>Cons</th>
<th>Dosing</th>
</tr>
</thead>
<tbody>
<tr>
<td>35. Published method (Zmurko et al. 2016)</td>
<td>39. Potentially fatal adverse events</td>
<td>45. 5-10 mL/kg/day is recommended, maximum 50 mL/kg/day (Diehl et al. 2001; Turner et al. 2011)</td>
</tr>
<tr>
<td>36. Slower absorption of drug</td>
<td>40. Requires “moderate technical skill and confidence” (Turner et al. 2011)</td>
<td>Allows 40-80 mg/kg/day of 7d2CmA</td>
</tr>
<tr>
<td>37. Simulate human dosing – not primary goal here</td>
<td>41. Lower bioavailability (51% of IV route for 7d2CmA)</td>
<td>In rats, 20 mg/kg dose gives 0.2 μM 7d2CmA in plasma after 24 hrs</td>
</tr>
<tr>
<td>38. More forgiving of irritating or impure drug preparations</td>
<td>42. First-pass metabolism significant for 7d2CmA</td>
<td>46. 0.5% carboxymethylcellulose is too much, use 0.2% (Zmurko et al. 2016)</td>
</tr>
<tr>
<td></td>
<td>~100x more drug in liver than blood (Olsen et al. 2004)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>43. Variable uptake and metabolism based on feeding/drinking/sleeping times</td>
<td></td>
</tr>
<tr>
<td></td>
<td>44. High volumes may be forced into lungs or into intestines</td>
<td></td>
</tr>
</tbody>
</table>

### Subcutaneous Injection

<table>
<thead>
<tr>
<th>Pros</th>
<th>Cons</th>
<th>Dosing</th>
</tr>
</thead>
<tbody>
<tr>
<td>47. Simple to perform (Turner et al. 2011)</td>
<td>50. Irritating substances can cause abscesses.</td>
<td>51. 5-10 mL/kg/day recommended (Diehl et al. 2001; Turner et al. 2011)</td>
</tr>
<tr>
<td>48. Avoids first-pass metabolism</td>
<td></td>
<td>40 mL/kg/day maximum</td>
</tr>
<tr>
<td>49. Slower absorption rate</td>
<td></td>
<td>40-320 mg/kg/day of 7d2CmA</td>
</tr>
<tr>
<td>Can also use oily “depots” for extended release.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(Table B-2, page 1)
### Intravenous Injection

<table>
<thead>
<tr>
<th>Pros</th>
<th>Cons</th>
<th>Dosing</th>
</tr>
</thead>
<tbody>
<tr>
<td>52. 100% drug delivery</td>
<td>54. Instant drug delivery, no extended uptake</td>
<td>56. 5 mL/kg/day recommended (Diehl et al. 2001)</td>
</tr>
<tr>
<td>53. Avoids first-pass metabolism</td>
<td>55. Requires port or cannula for daily injections</td>
<td>Can go up to 25 mL/kg/day by slow infusion (5-10 min)</td>
</tr>
<tr>
<td></td>
<td>Good surgical skill required for long-term installation (Turner et</td>
<td>57. Can use pumps with a catheter</td>
</tr>
<tr>
<td></td>
<td>al. 2011)</td>
<td>58. Port (internal) or cannula (external)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Port preferred since cannot be chewed on</td>
</tr>
</tbody>
</table>

### Intraperitoneal Injection

<table>
<thead>
<tr>
<th>Pros</th>
<th>Cons</th>
<th>Dosing</th>
</tr>
</thead>
<tbody>
<tr>
<td>59. Simple to perform (Turner et al. 2011)</td>
<td>61. Relatively frequent complications limit use for repeated doses</td>
<td>63. 10-20 mL/kg/day recommended (Diehl et al. 2001; Turner et al. 2011)</td>
</tr>
<tr>
<td>60. Slower absorption rate, but faster than subcutaneous</td>
<td>(Diehl et al. 2001)</td>
<td>80-160 mg/kg/day of 7d2CmA</td>
</tr>
<tr>
<td></td>
<td>62. Subject to first-pass metabolism</td>
<td></td>
</tr>
</tbody>
</table>

(Table B-2, page 2)
<table>
<thead>
<tr>
<th><strong>Implantable Pumps</strong> (DURECT Corporation 2017b, a)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pros</strong></td>
</tr>
<tr>
<td>38. Maintain constant plasma concentration of drug</td>
</tr>
<tr>
<td>65. Limited by drug solubility</td>
</tr>
<tr>
<td>Programmable</td>
</tr>
<tr>
<td>Pumps would require refilling daily</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

(Table B-2, page 3)
Figure B-1: LRV-high and LRV-low parasites identified by flow cytometry.

The ‘No Primary’ peak (red) is parasites stained only with the secondary antibody and represents background staining by the secondary antibody. The ‘Untreated’ peak (black) is LRV+ parasites treated with DMSO rather than 2CMA. The ‘2CMA-Treated’ trace (blue) shows the distribution of LRV1 in a culture treated with 1 µM 2CMA for 4 passages.
Figure B-2: LRV1 levels during treatment with 1 µM 2CMA.

Plot shows the changes in LRV1 levels over the course of 4 passages in 1 µM 2CMA (n=1). Grey lines show the geometric mean of the LRV-high (▲) and –low (▼) parasite populations. Black line shows the proportion of LRV-low parasites in the culture (●).