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Antiviral screening identifies adenosine analogs targeting the endogenous dsRNA Leishmania RNA virus 1 (LRV1) pathogenicity factor

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The endogenous double-stranded RNA (dsRNA) 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 Leishmania braziliensis and Leishmania 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 capsid flow cytometry assay, we identified two 2′-C-methyladenosine 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 six cell doublings, consistent with a measured LRV1 copy number of about 15. Viral load 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 vivo studies of LRV1-mediated virulence.

Significance

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. As a first step toward the identification of therapeutic LRV1 inhibitors, we identified two adenine analogs that selectively inhibited LRV1 replication. These analogs were used as tools to confirm that viral inheritance is by random segregation, as well as to generate LRV1-cured lines of Leishmania guyanensis, which correspondingly lost the increased pathogenicity conferred by LRV1. These compounds hold promise as leads to ameliorate the severity of LRV1-bearing Leishmania infections, and raise the possibility of targeting other protozoal infections whose pathogenicity may be exacerbated by similar endogenous viruses.


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microbial sources including the microbiota or coinfections (23). Recent studies show that the presence of LRV1 in clinical isolates of *L. braziliensis* and *L. guyanensis* correlates with drug-treatment failure (17, 20), phenomena that could readily be explained by the increased parasite numbers 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 toward amelioration of disease pathology. As one approach, murine vaccination using the LRV1 capsid results in significant protection against LRV1+ *L. guyanensis* (25).

Here we describe a complementary approach, targeting LRV1 directly using small-molecule inhibitors. Although effective antivirals are available for many viral targets including retroviruses, DNA viruses, and single-strand RNA (ssRNA) viruses (26), little effort has gone into agents acting against dsRNA viruses (27). These comprise at least 10 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 and rotaviruses (Reoviridae) cause serious human disease. For protoviruses, their role in the exacerbation of human disease is only now beginning to be appreciated (6, 29). Because 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 antileishmanial agents in the treatment of ongoing infection.

As a starting point, we focused on nucleoside analogs, a class that includes many widely used and effective antivirals (Table S1) (26). Following uptake and activation to the triphosphate form, these analogs 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 antileishmanial purine analogs; however, whereas 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 antileishmanial purine analogs; however, whereas the nucleobase 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.

**Results**

*Measurement of LRV1 Levels by Capsid Flow Cytometry.* Because 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 antisera (35) followed by detection with Alexa Fluor488-conjugated goat anti-rabbit IgG. We found that fixation with 2% (wt/vol) formaldehyde followed by permeabilization with Triton X-100 yielded a clear LRV1-dependent profile (Fig. L4). Titration of the anticapsid antisera showed that dilutions around 1:16,000 gave a strong signal with excellent selectivity between LgyLRV1+ and LRV1− (Fig. L1B), with little background staining evident in immunofluorescence microscopy. Under these conditions and as seen in previous immunofluorescence studies (36), LgyLRV1+ showed a strong, homogeneous LRV1 distribution (Fig. L4). We attempted similar studies with anti-dsRNA antibodies (36), but were unable to identify fixation conditions that gave similarly clear discrimination between LgyLRV1+ and LRV1− by flow cytometry.

*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 *Leishmania* (Fig. S1 and Tables S1 and S2). These compounds were examined for their ability to inhibit the growth of LgyLRV1+ and virus levels by LRV1 capsid flow cytometry. LgyLRV1− parasites grew similarly to LgyLRV1+ 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 ~3 (Fig. 2; black or red dots within large dashed circles, Fig. S2, and Table S2). All nucleobase analogs fell within this group, as did foscarin (a structure analog of pyrophosphate). Within this group, a subset showed more than 10-fold inhibition of *L. guyanensis* growth (Fig. 2, red dashed circle and black dots above; Fig. S3 A and B; and Table S2), including known antileishmanials, such as allopurinol, mycophenolic acid, and 4-aminopyrazolopyrimidine (APP). Several additional compounds showed leishmanial inhibition at the concentration tested (Fig. 2, Fig. S2B, and Table S2); 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 (Fig. 2, Fig. S2A, and 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. S1A and Table S2). Both 2′-C-methyladenosine (2CMA) and 7-deaza-2′-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 μM. Both had previously been shown to inhibit the hepatitis C virus (HCV) RDRP following activation (37, 38). In contrast, 2′-C-methylcytidine or guanosine had little effect on LRV1 levels or L. guyanensis 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 mercaptopurine (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 or resistance to nucleoside hydrolases).

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

2CMA Preferentially Inhibits LRV1 Replication. Titrations were performed to quantify the potency of 2CMA and 7d2CMA against L. guyanensis 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 L. guyanensis 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 in inhibition (EC50 50–100 μM). Because the available quantities of 7d2CMA were limiting and both compounds were similarly selective for L. guyanensis, we focused thereafter on 2CMA.

Inhibition of 2CMA LRV1 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 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 showed similar inhibition of L. guyanensis growth and LRV1 levels, whereas at the highest concentration tested, allopurinol inhibited L. guyanensis 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 L. guyanensis growth or LRV1 capsid synthesis was seen with increasing concentrations of allopurinol (~3 μM) (Fig. S3D).
LRV1 Inhibition Is Independent of Leishmania Growth Inhibition. Agents inducing stress or growth arrest have been used to cure fungal Totiviridae, with cycloheximide (CHX) used often (44, 45). Growth of \textit{L. guyanensis} at 10 or 100 nM CHX resulted in an increase in population doubling time, from \textasciitilde 7.7 h to 11.2 or 44.7 h, 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−” parasite population seen at any CHX concentration (Figs. 4B and C). Similar results were obtained with clotrimazole, which inhibits \textit{Leishmania} growth through inhibition of sterol synthesis (Fig. 4D). Finally, no correlation was seen between LRV1 levels and growth rate in our test compound screening (Fig. 2 and Fig. S2) or exposure to hygromycin B (46). Thus, inhibition of \textit{Leishmania} growth alone does not alter LRV1 levels.

Viral Loss Occurs by Random Dilution. The availability of an inhibitor with strong selectivity for LRV1 over parasite growth provided an 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 to 8.5 h (Fig. 3). The average LRV1 levels immediately declined, with capsid and RNA levels falling in parallel (Fig. 5A and 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 and B). When visualized at the population level by flow cytometry, LRV1 capsid levels per cell declined homogeneously at every time point tested until only background staining was evident by six 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.

2CMA Induces LRV1− Populations. To explore the loss of LRV1 further, we performed a series of “washout” experiments, growing \textit{LglyLRV1+} in 100 μM 2CMA for one, three, four, or six cell doublings followed by transfer to drug-free media. After one doubling, a time when LRV1 levels had only decreased twofold, LRV1 capsid levels rapidly returned to WT levels and distribution. In contrast, when 2CMA was maintained for three or four 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 (Fig. 5 C and D). One population expressed LRV1 at levels similar to control \textit{LglyLRV1+}, whereas the other resembled \textit{LglyLRV1−} (Fig. 5D, Top and Middle). Parasites with \textit{LglyLRV1+} capsid levels were the majority (55%) in the three-doubling washout population, whereas these had declined to 36% percent in the four-doubling washout population (Fig. 5D). The \textit{LglyLRV1+} population increased from 31 to 50% of the total cell population during this time. Finally, after six cell doublings of growth with 2CMA, the LRV1 capsid profile was indistinguishable from that of the \textit{LglyLRV1+} and the six-doubling washout population revealed only parasites maintaining the \textit{LglyLRV1+ capsid}-staining profile (Fig. 5D). This population was maintained for at least six passages (\textasciitilde 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− population could not emerge after only three to six cell doublings (Fig. 5), roughly corresponding to copy numbers of 5–64 (2^5–2^6) and consistent with fraction of LRV1− cells emerging in the washouts (Fig. 5D). \textit{LglyLRV1+} copy number was previously estimated as 24–100 by competitive PCR assay (48). To assess LRV1 copy number independently in the clonal \textit{LglyLRV1+} line studied here, we isolated total RNA quantitatively from a known number of cells, and estimated LRV1 copy number by quantitative RT-PCR (qRT-PCR), using a standard curve established from a cloned LRV1 genome (Methods). This process yielded an estimated average LRV1 copy number of 15 ± 0.9 per cell (n = 3), consistent with range estimated from the rate of drug-induced loss above.

Second, 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-like population suggests that there may be a “set point” for LRV1 levels. Because 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.

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 \textit{L. guyanensis}. 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 of 30). However, if the population was allowed to grow in the absence of 2CMA for another \textasciitilde six 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 six exhibited LRV1 capsid levels/profiles identical to the \textit{LglyLRV1+} control, whereas two showed profiles identical to the \textit{LglyLRV1−} parent (representatives shown in Fig. 6B). Interestingly, four lines showed more complex profiles, with populations showing range of intensities...
spanning those from LRV1\textsuperscript{−} to LRV1\textsuperscript{+} 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\textsuperscript{+} and LRV1-cured lines that 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. 6C and D). These clones thus constituted matched WT and LRV1-cured lines appropriate for subsequent studies of LRV1 effects.

**LRV1 Correlates with Increased Cytokine Secretion and Mouse Infectivity.** With matched 2CMA-treated LRV1\textsuperscript{+} and LRV1\textsuperscript{−} (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 (BMM) in vitro, followed by assays for secretion of two characteristic LRV1-dependent cytokine reporters, IL-6 and TNF-\(\alpha\). Cytokine secretion induced by the LRV1\textsuperscript{+}2CMA-treated lines was comparable to that of the parental LgyLRV1\textsuperscript{+} line, whereas cytokine secretion induced by the 2CMA cured lines was considerably less, and comparable to that of the LgyLRV1\textsuperscript{−} control (Fig. 7A and 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\textsuperscript{−} dependency for both pathology and parasite abundance was observed in comparisons of the matched 2CMA-treated LRV1\textsuperscript{+} vs. LRV1\textsuperscript{−} (cured) lines (Fig. 7C and D). Importantly, the response to the 2CMA-treated LRV1\textsuperscript{−} lines closely matches that to the control parental LgyLRV1\textsuperscript{+} line and, similarly, the response to the 2CMA-treated LRV1\textsuperscript{−} line closely matches that to the LgyLRV1\textsuperscript{−} control (Fig. 7C and D), both of which were studied previously (7).

**Discussion**

In this study, we report the identification of compounds specifically targeting the LRV1 dsRNA virus of *L. guyanensis* and *L. braziliensis*, two representatives of the Totiviridae. Our findings have relevance for the specific therapeutic inhibition of *Leishmaniavirus*, basic studies of viruses within the Totiviridae, the development of antivirals 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 although these studies used it in a relatively low throughput manner, it should be scalable for higher throughput. The results were confirmed by anticapsid or anti-dsRNA Western or slot blotting, or quantitative RT-PCR (Fig. 6C and 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\textsuperscript{+} lines.

We focused on known antivirals for several reasons: first, despite significant advances in targeting many retroviruses, DNA viruses, or ssRNA 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, because 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 antileishmanial therapy. Several new compounds not previously reported to inhibit *Leishmania* were identified (Fig. 2 and Tables S1 and S2), but were not pursued further here.

We identified two compounds that showed preferential inhibition of LRV1, 2CMA, or 72CMA (Fig. 3 and Fig. 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 *LbrLRV1*, albeit with somewhat less selectivity over growth. The EC\textsubscript{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 HCV, where they target the viral RDRP by chain termination (37, 38, 49). By molecular modeling of the L. guyanensis 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 finding supports our working hypothesis that both anti-LRV1 compounds are activated to triphosphates, where they act to inhibit RDRP activity. These compounds represent the only inhibitors known to act against both LRV1 and L. guyanensis in our screen, because 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 three to six cell doublings following 2CMA treatment (Fig. 5). This finding implies the viral copy number was relatively low, less than 8–64 (2^3–4), 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. If this unexpectedly low value for LRV1 copy number applies generally to LRV1s in other Leishmania strains or species, it could provide

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.

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 and B). This finding 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 L. guyanensis in our screen, because without continued parasite growth LRV1 cannot be lost by dilution, and indeed may increase somewhat (Fig. 2).

Matched 2CMA-treated LRV1 and LRV1-cured lines recapitulate LRV1-dependent virulence. (A and B) Cytokine secretion by BM cells infected 24 h after infection with L. guyanensis LRV1 or LRV1cured lines (48) or footpad swelling (D) was measured at the peak of the infection (28 d). Each bar represents pooled data from eight mice total, four for each LgylRV1 and LgylRV1−/−. Para-site numbers (luminescence from luciferase reporter) (C) or footpad swelling (D) was measured at the peak of the infection (28 d). Each bar represents pooled data from eight mice total, four for each Lgyl line used. LRV1−/− (clones 10-9 and 10-10) and LRV1+ (clones 10-5 and 10-6) lines are shown; error bars represent ±SD. Data for control parasites are replotted from Ives et al. (7).
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 three to six cell doublings and a brief period of growth without drug, cultures manifested two distinct parasite populations by capsid flow cytometry: one similar to Lgy-LRV1+ and a second similar to Lgy-LRV1− (Figs. 5 and 6). The fraction of Lgy-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+ parasites, we adopted a protocol in which parasites were treated for only a brief period 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 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 and 4, Fig. S2, and Tables S1 and S2). 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+ variants might occur at a low frequency, which occasionally may emerge or be recovered by methods more sensitive than used here.

Antiviral Cures and the Generation of Isogenic LRV1− Lines for the Study of LRV1-Dependent Virulence. Treatment with 2CMA 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− 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.

The Potential for Antitoxoplasmal 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 antileishmanials. 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, further 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 from that used 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+ lines (7).

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

Methods

Parasites and Growth Media. Most studies were performed using luciferase-expressing transfectants of L. guyanensis (MHOM/BR/78/M4147) described previously [LRV1+ LgyM4147/SSU/RS2AT-LUC(b)c3 and LRV1− LgyM4147/pX63HYGSSU/RS2AT-LUC(b)c4] (54); these are termed LgyLRV1+ and LgyLRV1−, respectively. Two strains of L. braziliensis were examined: LEM2780 (MHOM/BO/90/CS) and LEM3874 (MHOM/BO/99/M7252 no. 3) (12). Parasites were grown in Schneider’s media (Sigma) prepared according to the supervisor’s instructions with 6.5% and supplemented with 0.76 mM hemin, 2 μg/mL bopterin, 50 U/mL penicillin, 50 μg/mL streptomycin, and 10% (vol/vol) heat-inactivated FBS. Cell concentrations were determined using a Coulter Counter (Becton Dickinson).

Drug-Inhibition Tests. Compounds were purchased or obtained as summarized in Table S1, and the structures of the two most active anti-LRV1 compounds are shown in Fig. S1. Most studies were performed using luciferase-expressing transfectants of L. guyanensis (MHOM/BR/78/M4147) described previously [LRV1+ LgyM4147/SSU/RS2AT-LUC(b)c3 and LRV1− LgyM4147/pX63HYGSSU/RS2AT-LUC(b)c4] (54); these are termed LgyLRV1+ and LgyLRV1−, respectively. Two strains of L. braziliensis were examined: LEM2780 (MHOM/BO/90/CS) and LEM3874 (MHOM/BO/99/M7252 no. 3) (12). Parasites were grown in Schneider’s media (Sigma) prepared according to the supervisor’s instructions with 6.5% and supplemented with 0.76 mM hemin, 2 μg/mL bopterin, 50 U/mL penicillin, 50 μg/mL streptomycin, and 10% (vol/vol) heat-inactivated FBS. Cell concentrations were determined using a Coulter Counter (Becton Dickinson).

LRV1 Capsid Flow Cytometry. For capsid flow cytometry, 107 cells were fixed at room temperature using 2% (wt/vol) paraformaldehyde (Thermo Fisher) in PBS for 2 min. They were then incubated in blocking buffer (10% vol/vol) normal goat serum (Vector Laboratories) and 0.2% Triton X-100 in PBS for 30 min at room temperature. Anti-LgyLRV1 capsid antibody (35) was added with 1:20,000 dilution) and incubated at room temperature for 1 h. After two washes with PBS, cells were resuspended in 200 μL PBS with Alexa Fluor488-labeled goat anti-rabbit IgG (Alexafluor, Invitrogen; 1:1,000; or Thermo Fisher; 1:2,000 dilution), and incubated 1 h at room temperature. After two additional washes with PBS, cells were subjected to flow cytometry, gating for single cells and side scatter and the data analyzed using CellQuest software (BD Bioscience).

RNA Purification, cDNA Preparation, and qRT-PCR. For RNA purification, 107 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 h at 37 °C and repurified using RSC-5 column purification (Zymo Research). cDNA was prepared using SuperScript III (Invitrogen) and random priming according to the manufacturer’s instructions with pH adjusted to 6.5 and supplemented with 0.76 mM hemin, 2 μg/mL bopterin, 50 U/mL penicillin, 50 μg/mL streptomycin, and 10% (vol/vol) heat-inactivated FBS. Cell concentrations were determined using a Coulter Counter (Becton Dickinson).
using the 60 °C. Data were analyzed using ABI 7000 SDS software (v1.2.3) and normalized with a housekeeping gene) with primers SMB5335 (5′-CTGACTGGAGCGGAGGTTAA) and SMB5336 (5′-CAAAAACCTCCTCCAGA), and a 100-bp fragment of KMP-1.1 (a Leishmania housekeeping gene) with primers SMB5358 (5′-GCTGTGAGGAGGTTACAAC) and SMB5359 (5′-GTGCTCTCTTACGCGG). The reaction used Power SYBR Green (Applied Biosystems) in an ABI Prism 7000. Initial denaturation was at 95 °C for 10 min followed by 40 cycles of amplification with 15 s at 95 °C, and 1 min at 60 °C for annealing using ABI 7000 SDS software (v1.2.3) and normalized using the ΔΔCT method. RNA slot blot analysis was performed as described previously (36).

The LV1 copy number per cell was estimated in comparison with a standard curve generated using DNA from a plasmid bearing the LRV1 capsid gene (p6670, pB5/KLV1-1) and the average yield of RNA per cell across multiple L. guyanensis RNA preparations (5.12 ± 1.17 μg/10^6 cells; n = 34).

**Isolation of LRV1** and **LRV1-1 Clonal Lines by Brief Treatment with 2CM.** LgLV1′ parasites were grown for one passage in media containing 25 μg/mL nourseothricin (Werner BioAgent) to verify the presence of the integrated luciferase (LUC) gene (54). Cells were then grown one passage in the absence of nourseothricin, and inoculated into Schneider’s media at a concentration of 2 × 10^4 cells/mL in media containing 10 μM 2CMa. Growth was measured and LV1 quantitated by flow cytometry. At various times, cells were either plated directly, or transferred to drug-free media, and allowed to grow an additional six cell doublings before 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.

**Macrophage Infections, Cytokine Assays, and Mouse Infection.** Infections of CS7BL/6 mouse bone marrow-derived macrophages 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-wk-old CS7BL/6 mice were purchased from Jackson Laboratories. Parasites were grown into stationary phase (2 full days) and 10^5 parasites were injected on the plantar aspect of the left foot. Foot swelling was measured 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 v2.60 (Perkin-Elmer).

**Statement Identifying Institutional and 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 Swiss Federal Veterinary Office 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 National Institutes of Health (61). 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 Accreditation and Certification of Laboratory Animal Care International.

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