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Concentration of 2′C-methyladenosine triphosphate by Leishmania guyanensis enables specific inhibition of Leishmania RNA virus 1 via its RNA polymerase

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Edited by Charles E. Samuel

Leishmania is a widespread trypanosomatid protozoan parasite causing significant morbidity and mortality in humans. The endobiont dsRNA virus Leishmania RNA virus 1 (LRV1) chronically infects some strains, where it increases parasite numbers and virulence in murine leishmaniasis models, and correlates with increased treatment failure in human disease. Previously, we reported that 2′C-methyladenosine (2CMA) potently inhibited LRV1 in Leishmania guyanensis (Lgy) and Leishmania braziliensis, leading to viral eradication at concentrations above 10 μM. Here we probed the cellular mechanisms of 2CMA inhibition, involving metabolism, accumulation, and inhibition of the viral RNA-dependent RNA polymerase (RDRP). Activation to 2CMA triphosphate (2CMA-TP) was required, as 2CMA showed no inhibition of RDRP activity from virions purified on cesium chloride gradients. In contrast, 2CMA-TP showed IC50 values ranging from 150 to 910 μM, depending on the CsCl density of the virion (empty, ssRNA-, and dsRNA-containing). Lgy parasites incubated in vitro with 10 μM 2CMA accumulated 2CMA-TP to 410 μM, greater than the most sensitive RDRP IC50 measured. Quantitative modeling showed good agreement between the degree of LRV1 RDRP inhibition and LRV1 levels. These results establish that 2CMA activity is due to its conversion to 2CMA-TP, which accumulates to levels that inhibit RDRP and cause LRV1 loss. This attests to the impact of the Leishmania purine uptake and metabolism pathways, which allow even a weak RDRP inhibitor to effectively eradicate LRV1 at micromolar concentrations. Future RDRP inhibitors with increased potency may have potential therapeutic applications for ameliorating the increased Leishmania pathogenicity conferred by LRV1.

Leishmaniasis occurs in many regions of the world, with more than 12 million cases and more than 1.7 billion people at risk (1, 3). Three clinical presentations are most common: mild, self-healing cutaneous lesions (cutaneous leishmaniasis or CL),2 fatal visceral disease, and disfiguring metastatic forms such as mucocutaneous leishmaniasis (MCL) (4). The factors determining disease progression and responsiveness to treatment are unclear, but are thought to be both host- and pathogen-derived (5, 6).

Many isolates of Leishmania within the subgenus Viannia, including Leishmania braziliensis (Lbr) and Leishmania guyanensis (Lgy), bear Leishmaniavirus, a single-segmented dsRNA Totivirus known as Leishmania RNA virus 1 (LRV1) (5, 7–9). Like most other Totiviridae species, LRV1 is neither shed nor infectious and is inherited vertically (10, 11); indeed, phylogenetic evidence suggests that LRV1 strains have persisted and co-evolved with their Leishmania hosts over millions of years (10).

Previous work has established that mice infected with parasites containing the endobiont LRV1 exhibit greater pathology, higher parasite numbers, and increased metastasis (12, 13). These studies benefited from the availability of isogenic LRV1+ or LRV1− lines, generated spontaneously or by defined methods such as RNAi or antiviral drug treatment (14–16).

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 (17–19), whereas others do not (20, 21). These discrepant findings may be explained by other parasite or host factors known to contribute to MCL pathology (13, 22, 23). Furthermore, differences in the severity of disease are not always accurately captured by binary categorization as CL or MCL. Moreover, co-infections with viruses inducing Type I interferon responses exacerbate Lgy pathology and metastasis (24, 25), potentially obscuring the contributions of LRV1. Importantly, the presence of LRV1 in clinical isolates of Lbr or Lgy correlates with drug treatment failure and relapses (18, 20), which could be explained by the increased parasite numbers or altered host responses predicted from animal models (12, 13).

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The neglected tropical disease leishmaniasis is caused by species of the genus Leishmania, which are single-celled eukaryotic parasites transmitted by phlebotomine sand flies (1, 2).

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2 The abbreviations used are: CL, cutaneous leishmaniasis; MCL, mucocutaneous leishmaniasis; Lgy, Leishmania guyanensis; Lbr, Leishmania braziliensis; LRV1, Leishmania RNA virus 1; 2CMA, 2′-C-methyladenosine; 2CMA-TP, 2′-C-methyladenosine triphosphate; RDRP, RNA-dependent RNA polymerase; 7d2CMA, 7-deaza-2′-C-methyladenosine; LD, low-density; MD, medium-density; HD, high-density.

The neglected tropical disease leishmaniasis is caused by species of the genus Leishmania, which are single-celled eukaryotic parasites transmitted by phlebotomine sand flies (1, 2).
2′-C-methyladenosine triphosphate inhibits LRV1 RDRP

Figure 1. Leishmania LRV1 replication cycle and inhibitors. A, a schematic depiction of the LRV1 lifecycle. RNAs are indicated in color (+ strand, red; −strand, blue); the dsRNA genome within the mature virion is shown as straight lines, whereas ssRNAs are shown as jagged lines. The viral RDRP (black trapezoid) is shown fused to a capsid monomer (white circle), as the RDRP is generated through frameshift translation (27–29). B, chemical structures of adenosine, 2′-C-methyladenosine, and 7-deaza-2′-C-methyladenosine.

Overall, there is good reason to postulate a role for LRV1 in increasing disease severity in human leishmaniasis (13), although many questions remain.

LRV1 follows a typical totiviral life cycle where the dsRNA viral genome encodes two large overlapping reading frames, the capsid and RNA-dependent RNA polymerase (RDRP) (Fig. 1A). First, the dsRNA genome is transcribed by the viral RDRP into positive-sense ssRNA. As in many totiviruses, the RDRP is translated via a +1 frameshift, generating a capsid–RDRP fusion (27–29). The capsid monomers then self-assemble into immature virions (30), incorporating the positive-sense ssRNA transcript, which the RDRP then replicates into the mature dsRNA genome.

Vaccination of mice using the LRV1 capsid results in significant protection against LRV1+ Lgy strain M4147 LRV1 virions purified by CsCl equilibrium gradient centrifugation (7, 33). After fractionation, virions were detected and quantified by their reactivity with an anti-capsid antibody. We reproducibly observed three overlapping “peaks,” designated low-, medium-, and high-density (LD, MD, and HD) (Fig. 2). 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 (34, 35). 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) (36). Preliminary data from S1 nuclease digestion of viral RNA from these fractions were consistent with these assignments.

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 other three nucleoside triphosphates for 1 h, a time sufficient for one round of viral genome replication (33). RNA was purified and separated by native gel electrophoresis, and the products were visualized and quantified. Two products were always found: one about 5 kb, presumably corresponding to the full-length LRV1 genome, and smaller, heterogeneous products ranging from 0.1
to 0.5 kb, which we considered abortive transcripts (Fig. 3A). Neither extending the incubation time nor increasing the concentration of UTP significantly altered the profile obtained. Importantly, neither full-length nor small products were produced by corresponding preparations from LRV1-negative parasites (Fig. 3B).

**2CMA-TP specifically inhibits viral RDRP activity**

Incubation of the three LRV1 fractions with 2CMA-TP reduced synthesis of both the full-length and small RDRP products (Figs. 3–5). The synthesis of each product was quantitated and normalized to that obtained with drug-free controls. IC\(_{50}\) values were calculated by fitting the inhibition data with a logistic dose-response curve (Table 1). These ranged from 150 μM for full-length product synthesis by LD virions to 910 μM for small products synthesized by HD virions (Table 1, Figs. 3 and 4). These IC\(_{50}\) values were unexpectedly high, greatly exceeding the extracellular concentration of 2CMA shown previously to cause 50% inhibition of LRV1 abundance (~3 μM) (15). This did not arise artificially from 2CMA-TP degradation during the...
2′-C-methyladenosine triphosphate inhibits LRV1 RDRP

Table 1

Effect of 2CMA-TP on Lgy LRV1 RDRP activity

<table>
<thead>
<tr>
<th>Product Type</th>
<th>Low-density (n = 3)</th>
<th>Medium-density (n = 3)</th>
<th>High-density (n = 4)</th>
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<tr>
<td>Full-length</td>
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<tr>
<td>Small</td>
<td>150 (90–210)</td>
<td>250 (100–400)</td>
<td>420 (310–540)</td>
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<td>(n = 3)</td>
<td>(n = 3)</td>
<td>(n = 4)</td>
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Assay, as HPLC tests of RDRP reactions showed only 3.7 ± 0.6% (n = 3) loss of 2CMA-TP.

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. 1B), failed to inhibit RDRP activity at the highest concentration tested (600 μM; Fig. 5); nor in limited tests did 600–1000 μM TTP inhibit.3 These data show that high concentrations of a nonsubstrate nucleoside triphosphate do not inhibit RDRP activity nonspecifically. Together, these data suggest that despite its relatively low potency, under these assay conditions 2CMA-TP inhibition of LRV1 RDRP activity was specific.

2CMA activation to 2CMA-TP within parasites

To account for the relative sensitivity of Lgy LRV1 within cells to 2CMA treatment compared with the insensitivity of LRV1 RDRP activity to inhibition by 2CMA-TP, we hypothesized that parasites must acquire and activate 2CMA, accumulating high 2CMA-TP concentrations. We developed an HPLC protocol capable of resolving synthetic 2CMA-TP from natural ribonucleotides and the internal standard, dGTP (Fig. 6, A and B; a small peak presumed to be 2CMA-DP was also observed). Although dGTP occurs naturally, its intracellular concentration of ~5 μM is well below the limit of detection for this assay and thus does not interfere with its use for this purpose (37). Standard mixtures were then used to generate a calibration curve relating peak area to 2CMA-TP amount.

We next evaluated several protocols for extracting parasite nucleotides and determined that extraction with 1:1 acetonitrilewater performed best, as judged by recovery of the added dGTP standard (“Experimental procedures”). We then compared the nucleotide profiles of LRV1 + Lgy grown in the presence or absence of 10 μM extracellular 2CMA for 20 h, a time corresponding to more than 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). This established the parasite’s capacity to activate 2CMA to 2CMA-TP.

Time and external 2CMA concentration dependence of 2CMA-TP accumulation

Leishmania cell volumes vary somewhat depending on the culture’s growth phase (38). To calculate the intracellular 2CMA-TP concentration, we therefore determined the average volume of Lgy parasites under our assay conditions. In logarithmic growth phase, parasite volumes averaged about 23 femtoliters (“Experimental procedures”).

We measured the intracellular concentration of 2CMA-TP as a function of time of incubation with 10 μM external 2CMA. Within 1.5 h, 2CMA-TP levels had risen to concentrations comparable with the minimal RDRP IC₅₀ levels (Fig. 7A), and had reached maximal levels by 8 h.

We then measured the intracellular 2CMA-TP levels following growth in varying levels of 2CMA for 20 h (Fig. 7B). The concentrations were chosen to span a range from noninhibitory (1 μM) to that sufficient to eradicate LRV1 entirely (10 μM) (15). This range of concentrations, internal 2CMA-TP levels exceeded external 2CMA concentrations by 40–80-fold (Fig. 7B).

When propagated in the subinhibitory dose of 2CMA (1 μM), the intracellular 2CMA-TP concentration was 78 ± 9 μM (Fig. 7B, n = 4), well below the minimal IC₅₀ for RDRP inhibition (150 μM for full-length products from LD virions; Table 1). When treated with 3 μM external 2CMA, the EC₅₀ for LRV1 inhibition, intracellular 2CMA-TP levels rose to 152 ± 43 μM (n = 4), comparable with the minimal RDRP IC₅₀. Finally, cells propagated in 10 μM external 2CMA reached an intracellular 2CMA-TP concentration of 410 ± 110 μM (n = 4) (Fig. 7B), well in excess of the minimal IC₅₀ for RDRP inhibition. Thus, with increasing external 2CMA, the steady-state levels of intra-
Parasites retain 2CMA-TP for an extended period following 2CMA removal

Once phosphorylated by cells, some nucleoside analogs are retained for an extended period as triphosphates, thereby contributing significantly to drug efficacy (39). To assess this, Lgy parasites were incubated for 20 h in 10 μM 2CMA, washed, and resuspended in drug-free medium, after which intracellular 2CMA-TP rose progressively to values exceeding the minimal RDRP IC50, which in turn corresponded reasonably well to the observed effects on LRV1 inhibition.

**Parasites retain 2CMA-TP for an extended period following 2CMA removal**

2CMA-TP levels were measured and corrected for parasite replication (Fig. 7C). Even after 8 h, 2CMA-TP levels remained at or above the minimal RDRP IC50. Indeed, the persistence of intracellular parasite 2CMA-TP greatly exceeded that of serum 2CMA measured in mammalian models (40).

**A quantitative simulation of RDRP and virus inhibition**

We asked whether a simple computational model using the relative rates of parasite and viral replication under conditions of drug treatment could quantitatively simulate the experimental data above. We developed a model employing Gibson and Bruck’s next reaction modification to Gillespie’s stochastic simulation algorithm (41), which directly simulates the occurrence of individual events over time by picking the next event time from an associated probability distribution (“Experimental procedures”; supporting File S2).

In this model, the relevant parameters were parasite and virus replication rates. We used the experimentally-measured parasite population doubling time of 7.5 h and assumed that in the absence of drug pressure the relative parasite and virus replication rates were identical. Simulations began with an initial population of 1000 cells, a number large enough that repeated simulations deviated <1% from each other. The simulated parasites each initially contained 16 LRV1 particles, consistent with prior measurements of virus numbers (15). Using these conditions, the simulation correctly maintained the LRV1 population over time at an average of 16 virions per parasite in the absence of drug pressure (Fig. 8).

The effect of 2CMA was then modeled by adjusting the ratio of LRV1 to parasite replication rates (Rv:Rp). Importantly, at concentrations relevant to these studies, 2CMA has little effect on the growth of the parasite itself (15). After setting Rv:Rp to 1:2, 1:3, or 1:4, LRV1 viral loss during 2CMA treatment was shown to be significantly inhibited (Fig. 8). Comparison of the simulations with Rv:Rp of 1:3 or 1:4 showed close correspondence with the experimentally-measured rate of LRV1 loss.
under 2CMA inhibition (15) (Fig. 8; solid lines, simulation; dashed lines, experimental).

To compare these predictions with our experimental data on parasite replication and RDRP inhibition, we made an initial simplifying assumption that the primary determinant of LRV1 replication would be the lowest RDRP 2CMA-TP IC₅₀ value (150 μM, for full-length product synthesis by LD virions; Table 1; Fig. 4A). Because RDRP inhibition would reduce viral RNA levels within infected cells, RDRP activity would likely become rate-limiting at high 2CMA-TP concentrations. We then calculated the 2CMA-TP concentrations required to give Rₚ:R₀ of 1:2, 1:3, or 1:4. Importantly, at 10 μM extracellular 2CMA, intracellular 2CMA-TP is 410 μM, and Rₚ:R₀ was calculated to be ~1:3 (Fig. 8). Thus the simulation and experimental data are in good agreement about the quantitative and qualitative aspects of RDRP inhibition and viral loss. This suggests that despite the potential complexity of viral replication, the system behaves as RDRP activity is rate-limiting for viral replication and that our in vitro measurements of 2CMA-TP inhibition are consistent with our in vivo measurements of viral elimination time courses.

Discussion

Previously, we showed that two 2’-C-methyladenosine analogs selectively inhibit the replication of Lgy and Lbr LRV1 but have significantly less effect on the parasite growth rates. These compounds were the first such inhibitors to be described for any totivirus (15). There, 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. Importantly, the ability of the parasite to accumulate high levels of 2CMA-TP (Fig. 7) was strongly correlated with viral elimination at concentrations above 3 μM external 2CMA (15). These data provide key information for the design of other improved LRV1 inhibitors with greater potential for use as therapeutics.

We first established an assay for RDRP activity from purified virions, separated on CsCl gradients to obtain virions in different stages of maturation (Figs. 1–3). RDRP activity depended 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 the IC₅₀ values for full-length product synthesis were somewhat lower than those measured for small product synthesis (p < 0.05), and significantly less for low-density virions than high (p < 0.001; Table 1). These may reflect the intrinsic sensitivities of the RDRP activity within mature and immature viral particles, perhaps related to the distinct transcriptase/positive-strand (mRNA) and replicase/negative-strand synthesis activities. This is the first such report for totiviruses, for which antiviral drugs have only recently been reported (15).

Differential effects on replicase versus transcriptase activity have also been seen in reoviruses, where ribavirin triphosphate inhibits replicase but not transcriptase activity (42). Our studies were constrained by two factors: first, the virion-based RDRP assay depends on native RNA substrates, and second, the virion-containing fractions used were not homogeneous (Fig. 2). This prevented clean separation of RDRP transcription and replication activities, and precluded the use of tightly-controlled initiation and elongation assays, required for determination of the precise mechanism of RDRP inhibition by 2CMA-TP. Future studies with purified RDRP and defined RNA substrates will be required to fully elucidate the mechanism of action of 2CMA-TP.

2CMA was completely inactive for RDRP inhibition, as were dATP or TTP, structurally similar nonsubstrate nucleoside triphosphates (Fig. 5). Activation to triphosphate form is common and often rate-limiting among nucleoside analog drugs (43–45). It was also shown previously that the triphosphate form of 2CMA, but not the analog itself, is active against the hepatitis C virus RNA polymerase (46).

Despite the ability of 2CMA to clear LRV1 from parasites grown in micromolar concentrations of drug (15), 2CMA-TP inhibition of LRV1 RDRP activity was not very potent, with IC₅₀ values ranging upwards of 150 μM (Table 1). We resolved this discrepancy by showing that the parasites avidly scavenged 2CMA from the medium and efficiently converted it to the active triphosphate form (Fig. 6), reaching intracellular 2CMA-TP concentrations more than 40-fold that of extracellular 2CMA (Fig. 7). This likely reflects the fact that as purine auxotrophs, Leishmania parasites must avidly scavenge all naturally occurring purines from their environment, through a combination of powerful transporters and metabolic interconversions (32). One particularly important step for 2CMA and related inhibitors may be adenosine kinase (47, 48), which may mediate the initial and often rate-limiting phosphorylation of antiviral nucleosides (44, 49). Strong accumulation of toxic anti-leishmanial purines has also been noted in earlier studies (50, 51). Thus the salvage pathway converts 2CMA into a potent tool for eliminating the virus.

Importantly, the accumulated levels of intracellular 2CMA-TP closely matched the consequences of RDRP inhibition and LRV1 loss. When grown in 10 μM external 2CMA, internal 2CMA-TP levels greater than 400 μM were attained, well over the minimal LRV1 RDRP IC₅₀ (Table 1). 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 (15). These experimental observations were corroborated using Gillespie (52) simulation to model LRV1 loss (Fig. 8). Our studies did not examine other potential 2CMA-TP targets such as the capsid endonuclease (53–57) and could not discern the exact mechanism of RDRP inhibition by 2CMA-TP. Nevertheless, these analyses show that the elimination of LRV1 by 2CMA can be largely explained through the direct inhibition of LRV1 RDRP activity by 2CMA-TP.

Previously, we proposed that treatments targeting LRV1 could be used therapeutically to ameliorate the severity of Lgy and Lbr infections (15, 31). 2CMA-TP accumulates rapidly to inhibitory concentrations within 1–2 h of 2CMA exposure, and once formed, is retained at inhibitory concentrations for >8 h (Fig. 7). This suggests that a relatively short period of treatment may lead to a prolonged period of viral inhibition. In animal models, 2CMA has a short serum half-life, which precludes its use in humans or animals. The serum half-life of 7d2CMA,
2′-C-methyladenosine triphosphate inhibits LRV1 RDRP

however, is markedly longer (0.3 versus 1.6 h (58)). Interestingly, 7d2CMA shows an EC_{50} against Zika virus in cultured mammalian cells of about 10 μM, and dosing regimens have shown significant inhibition in animal models (59, 60). This suggests it might likewise be possible to achieve inhibition of LRV1 in vivo using 7d2CMA.

In future work, one priority will 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 (15), suggesting that the lack of activity may reflect failure to inhibit the LRV1 RDRP itself rather than insufficient activation to triphosphates. Simil

larly, we found that several immucillins shown previously to inhibit Leishmania nucleoside hydrolases (Immmucillin A, DADMe-Immmucillin A, Immmucillin H, and DADMe-Immmucillin H (61, 62)) had little effect on the potency of 2CMA. While these immucillins are nucleoside analogs, they showed no inhibition of LRV1 levels when tested at concentrations up to 100 μM.

Additional priorities will be identifying compounds with more favorable pharmacokinetics and minimal toxicity against the mammalian host cell. Thus, efforts focusing on improved potency against the RDRP activity itself may prove most fruitful. Our work now sets the stage for future studies exploring the possibility that improved LRV1-targeted therapies may ameliorate the pathology of those Leishmania species and strains that bear this fascinating virus.

Experimental procedures

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 (63). 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) was prepared following the manufacturer’s instructions, supplemented with 10% heat-inactivated FBS, 0.76 mM hemin, 2 μg/ml of biopterin, 50 units/ml of penicillin, and 50 μg/ml of 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 of biotin, 5 μg/ml of hemin, 2 μg/ml of biopterin, 50 units/ml of penicillin, 50 μg/ml of streptomycin, and 40 mM HEPES, pH 7.4 (64). 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 (BD Bioscience).

Purification and fraction of virions on CsCl gradients

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 mM NaCl). Cells were then resuspended in 1 ml of ice-cold lysis buffer (TMN buffer plus 1 mM DTT, 1 M Complete protease inhibitor mixture (Roche Applied Science) 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 min. Lysis was completed by passing the mixture repeatedly through a 27-gauge needle, after which it was clarified by centrifugation at 15,000 × g for 10 min 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 ~72 h. 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% nonfat powdered milk in PBS) for 1 h, then stained with 1:2500 rabbit anti-capsid antibody (65) in blocking buffer plus 0.2% Tween 20 (TWEEN buffer) for another hour. The membrane was then washed 3 times for 5 min in 1 × PBS plus 0.1% Tween 20 (PBST). Membranes were next incubated in Tween buffer for 1 h with 1:10,000 goat anti-rabbit antibodies conjugated to IRDye 680 (LiCor Biosciences). Finally, the membranes were washed 3 times in PBST and once in PBS. Membranes were scanned with an Odyssey IR 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 (66). 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.

RDRP assay

RDRP activity of purified virions was measured using an [α-32P]UTP incorporation assay described previously (33). Briefly, 20-μl reactions contained 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 3 mM MgCl_2, 4 mM DTT, 50 μM each ATP, CTP, and GTP, 20 μCi of [α-32P]UTP, and 10 μl of virions. Reactions were incubated at room temperature for 1 h and quenched by addition of 350 μl of 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, whereas the radiolabeled products were detected by exposing an imaging plate for 24 h 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 (67). 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 Bio-
2′-C-methyldapenosine triphosphate inhibits LRV1 RDRP

science, 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 nonspecifically 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.

To estimate IC_{50} concentrations for each virion population and RDRP product, the quantified RDRP activity data were normalized to untreated and LRV – controls and fitted to logistic dose-response models. Fitting was performed and 95% confidence intervals were estimated using the “drc” package in the R statistical language (68).

Measurement of parasite volumes

Cultures of WT or GFP-expressing LRV1 + Lgy M4147 were seeded at 2 × 10^5 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, whereas another was immobilized by treatment with 20 mM sodium azide and imaged by spinning-disk confocal microscopy (69). Cell volumes were calculated using a custom ImageJ script (67) (supporting File S1). A standard curve relating forward scattering intensity to measured volume was developed from these data. Forward scattering intensity of parasites in our assay conditions was then measured and used to estimate the intracellular parasite volume.

Nucleotide extraction from Leishmania parasites

For each sample, 10^8 cells were collected by centrifugation at 2,200 × g, 4 °C for 5 min, re-suspended in 1 ml of ice-cold PBS and re-centrifuged. The cell pellet was gently re-suspended in 100 μl of ice-cold 0.5× PBS plus 7 nmol of dGTP as a recovery and elution standard. Cells were immediately lysed by rapidly re-suspending in 900 μl of ice-cold 5:4 acetonitrile:water mixture (70) and vortexing continuously for 5 min at 4 °C. Insoluble debris was pelleted at 16,000 × g for 5 min 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 of distilled water, flash frozen, and stored at −80 °C prior to HPLC analysis.

HPLC separation of nucleotides

Cell extracts were clarified by centrifuging for 2 min at 16,000 × g. Nucleoside di- and triphosphates were separated by isocratic HPLC as previously described (71). Briefly, a 20-μl aliquot of clarified extract was injected onto a Zorbax SB-C18 column (5 μm particle size, 250 × 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 (Fig. 6). 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 diphosphate 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). Varying amounts of ATP, 2CMA-TP, and dGTP standards were used to construct a calibration curve relating peak areas to compound amounts. Peak areas varied linearly with nucleotide amounts injected down to 50 pmol.

Gillespie simulation of LRV1 inhibition

We modeled the effects of 2CMA treatment on LRV1 using the next-reaction modification to the Gillespie algorithm (a more detailed description as well as code is provided in supporting File S2) (41). 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 (41, 52). At each step, the event with the shortest time remaining was selected, the simulation time incremented, and the model updated accordingly.


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References


Concentration of 2′C-methyladenosine triphosphate by *Leishmania guyanensis* enables specific inhibition of *Leishmania* RNA virus 1 via its RNA polymerase

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