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Short communication

# Lactimidomycin is a broad-spectrum inhibitor of dengue and other RNA viruses

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ABSTRACT

Dengue virus, a member of the *Flaviviridae* family, is a mosquito-borne pathogen and the causative agent of dengue fever. Despite the nearly 400 million new infections estimated annually, no vaccines or specific antiviral therapeutics are currently available. We identified lactimidomycin (LTM), a recently established inhibitor of translation elongation, as a potent inhibitor of dengue virus 2 infection in cell culture. The antiviral activity is observed at concentrations that do not affect cell viability. We show that Kunjin virus and Modoc virus, two other members of the Flavivirus genus, as well as vesicular stomatitis virus and poliovirus 1, are also sensitive to LTM. Our findings suggest that inhibition of translation elongation, an obligate step in the viral replication cycle, may provide a general antiviral strategy against fast-replicating RNA viruses.



Dengue virus 2 (DENV2), a member of the *Flaviviridae* family, is an enveloped, positive-strand RNA virus and the causative agent of dengue fever. Dengue infection can be serious, potentially leading to hemorrhagic fever, shock syndrome, and death. It is estimated that over 350 million people are infected annually and a third of the world's population is at risk (Bhatt et al., 2013). Despite these staggering numbers, there is currently no vaccine or antiviral drug available to prevent or to treat infection. The development of vaccines has been challenging due to the diversity of DENV serotypes and the occurrence of antibody-dependent enhancement of infection, a phenomenon in which neutralizing antibodies against one DENV serotype can exacerbate disease upon subsequent infection with another serotype (Guzman et al., 2013; Murphy and Whitehead, 2011).

Research and development of antivirals to fight DENV are therefore of great interest. Due to the intrinsically high mutation rate of RNA viruses, resistance to antiviral drugs that act against viral targets (*e.g.*, inhibitors of viral proteases and polymerases) can occur rapidly. To complement traditional antivirals, agents that act via host targets and that present higher barriers to resistance have become of increasing interest (for review see (Noble et al., 2010)). Since the immune system clears DENV and other acute viral pathogens if given sufficient time, the goal of antiviral therapy against these pathogens may be to shorten the duration of the infection and decrease viral burden by inhibiting replication, thereby reducing transmission and the incidence of severe disease. Celgosivir and other inhibitors of host alpha-glucosidases are examples illustrating the potential of this strategy. These compounds potently inhibit DENV replication, reduce disease, and improve survival in murine models (Perry et al., 2013; Rathore et al., 2011; Watanabe et al., 2016, 2012; Whitby et al., 2005); moreover, the genetic barrier to resistance against one such inhibitor, UV-4B, appears to be high (Plummer et al., 2015). Celogosivir's safety was demonstrated in a phase Ib trial (Low et al., 2014), and a new phase Ib/2a trial (NCT02569827) has been approved to investigate efficacy using an altered dosing regimen. Heralded by this work, additional strategies for inhibiting DENV and other RNA viruses via host targets through repurposing of known drugs or validation of new targets and antiviral entities are of considerable interest.

All viruses lack their own translational apparatus and rely entirely upon the host cell's protein synthesis machinery. Indeed, André Lwoff noted the absence of ribosomes and the cellular translation machinery as a defining feature of viruses (Lwoff, 1957).

*Abbreviations:* DENV2, dengue virus 2; VSV, vesicular stomatitis virus; PV1, poliovirus 1; LTM, Lactimidomycin; CHX, Cycloheximide; MPA, mycophenolic acid; PFU, plaque-forming unit; FFU, focus-forming unit; EMCV, encephalomyocarditis virus.

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The translation process consists of three steps: initiation, elongation, and termination. The initiation step is highly regulated and leads to formation of an elongation-competent 80S ribosomal complex. For most cellular mRNAs, this process is dependent upon the presence of a 5′ cap on the mRNA. Binding of eIF4F to the 5′ cap enables recruitment of the 40S ribosomal subunit to the mRNA. This is followed by the highly regulated, sequential loss of eIF2-bound GDP, recruitment eIF5B-GTP and the 60S ribosomal subunit, and loss of eIF5B-GDP and eIF1A to yield an elongation-competent 80S ribosomal complex (for review (Jackson et al., 2010)).

While some viruses accomplish translation initiation via cap-dependent mechanisms, they do so via a myriad of mechanisms with varying utilization of host eukaryotic initiation factors (eIFs). Other viruses initiate at internal ribosome entry sites (IRES) via cap-independent mechanisms (for review see (Walsh et al., 2013)). The elongation step results in polymerization of amino acids to synthesize polypeptides as templated by the mRNA template. Elongation requires (1) delivery of the correct aminoacyl-tRNA to the A-site by eEF1A; (2) formation of the new peptide bond, which transfers the nascent peptide to the A-site tRNA; and (3) eEF2-catalyzed transfer of this new peptidyl-tRNA to the P-site and transfer of the deacetylated tRNA to the E site, thereby freeing the A-site for the next aminoacyl-tRNA (Richter and Coller, 2015; Schneider-Poetsch et al., 2010b). All known viruses rely on cellular elongation factors for expression of the viral genome. For the *Flaviviridae* and other positive-strand RNA viruses, translation of the viral genome is an especially critical control point in the replication cycle. For DENV, translation efficiency has been shown to be a determinant of productive infection (Edgil et al., 2003).

Lactimidomycin (LTM) (Fig. 1A) is a natural product isolated from *Streptomyces amphibiosporus* (ATCC53964) that inhibits translation elongation through binding to the ribosome E-site. This prevents the ribosome from leaving the start site and blocks the very first round of elongation (Ju et al., 2005; Schneider-Poetsch et al., 2010a,b). Cycloheximide (CHX) also blocks translation elongation by binding in the E-site, but its smaller size permits binding of one tRNA in the E-site before elongation is halted. The absolute dependence of viruses upon translation elongation has stimulated interest in this process as a source of antiviral targets. Studies performed in the 1980s examined the use of cycloheximide as an inhibitor for encephalomyocarditis virus and vesicular stomatitis virus (VSV) (Ramabhadran and Thach, 1980; Yau et al., 1978); however, CHX's effects on transcription along with other toxic effects have been prohibitive for its development as a drug. LTM inhibits translation elongation with ten-fold greater potency than CHX while lacking CHX's effects on transcription even at high concentrations (Schneider-Poetsch et al., 2010a). This has stimulated considerable interest in evaluating LTM and related glutarimides as anticancer agents (Larsen et al., 2015; Micoine et al., 2013) and prompted us to examine LTM's potential as an anti-DENV agent.

Huh7 cells infected with DENV serotype 2 New Guinea C (DENV2 NGC) at a multiplicity of infection (MOI) of 1 were treated with varying concentrations of LTM for twenty-four hours, corresponding to a single round of infection (Fig. 1B). The cytotoxicity of LTM was evaluated in parallel to control for potential indirect antiviral effects due to a decrease in cell viability. LTM induced a clear dose-responsive inhibition of DENV2 infectious particle production (Fig 1B black circles) with an $EC_{90}$ value – defined as the concentration of inhibitor needed to reduce the single-cycle viral yield by 10-fold – of 0.4 μM, as determined by non-linear fit of the data. No measurable decrease in cell viability was detected at concentrations up to 12.5 μM (Fig 1B red triangles). While statistically significant cytotoxicity ($p < 0.05$) was observed in both

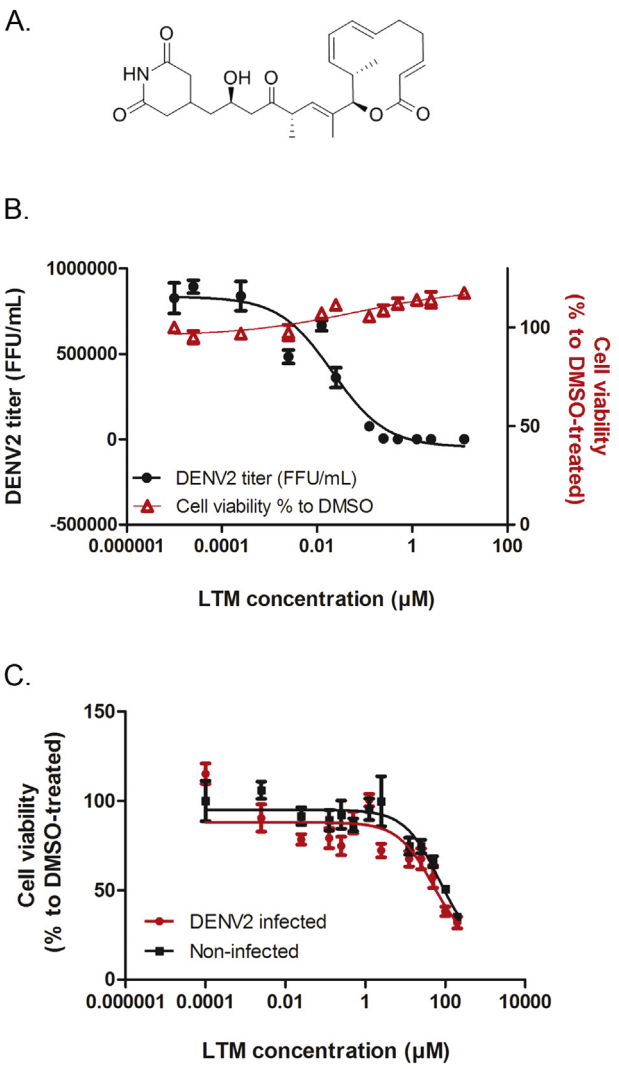


**Fig. 1.** Lactimidomycin inhibits DENV2 production in Huh7 cells at concentrations that are non-cytotoxic. (A) Structure of lactimidomycin (LTM). (B) LTM reduces DENV2 replication in Huh7 cells infected at an MOI of 1 (black dots) within 24 h without affecting cell viability (red open triangles). (C) Concentration-dependent effect of LTM on viability of DENV2-infected (red dots) and non-infected (black dots) Huh7 cells treated for 24 h post-infection. Viral titers were determined by focus-forming assay (see Supplemental methods), and cell viability was assessed by Cell Titer-Glo® assay. Experiments were performed in duplicates, and the error bars represent the mean ± SD.

DENV2-infected and non-infected cells at LTM concentrations above 25 μM (Fig. 1C), we were unable to determine the $CC_{50}$ of LTM, as a lower plateau was not reached even at 200 μM.

To assess the effects of LTM on translation and replication of the DENV2 genomic RNA, we utilized a replicon system in which the viral structural proteins are replaced by a luciferase reporter, thus permitting analysis of viral RNA translation and replication in the absence of viral entry, assembly, and egress (Clyde et al., 2008). In this system, luciferase activity served as a readout for translation of the input replicon RNA at early times following electroporation (<24 h) and as a marker of both viral translation and replication of the viral RNA at later time points ((Carocci et al., 2015) and Supplemental Methods). LTM inhibited DENV2-$Fluc^{WT}$ translation at 0.5 μM, as evidenced by a profound decrease in luciferase activity at 6 h post-electroporation (Fig. 2A). In experiments utilizing infectious DENV2 NGC, delaying LTM treatment until 12 h post-