Official journal of the Spanish Society of Chemotherapy

Brief report

Revista Española de Quimioterapia
doi:10.37201/req/016.2023

Estela Giménez[1]
Manuel Guerreiro[2]
Roberto Gozalbo-Rovira[3]
Cristobal Aguilar[2]
Eliseo Albert[1]
José Luis Piñana[4]
Carlos Solano[4,5]
David Navarro[1,3]

In vitro assessment of the combined effect of letermovir and sirolimus on cytomegalovirus replication

[1]Microbiology Service, Hospital Clínico Universitario, INCLIVA Health Research Institute, Valencia, Spain.
[2]Hematology Service, Hospital Universitario y Politécnico "La Fe", Valencia, Spain.
[3]Department of Microbiology, School of Medicine, University of Valencia, Valencia, Spain.
[4]Hematology Service, Hospital Clínico Universitario, INCLIVA Health Research Institute, Valencia, Spain.
[5]Department of Medicine, School of Medicine, University of Valencia, Valencia, Spain.

Article history
Received: 2 February 2023; Revision Requested: 9 April 2023; Revision Received: 12 April 2023; Accepted: 8 May 2023; Published: 28 June 2023

ABSTRACT

Introduction. Letermovir (LMV) is used for prophylaxis of cytomegalovirus (CMV) reactivation and end-organ disease in adult CMV-seropositive allogeneic hematopoietic stem cell transplant recipients (allo-HSCT). In turn, sirolimus (SLM) which displays *in vitro* anti-CMV activity, is frequently employed for prophylaxis of Graft vs. Host disease in allo-HSCT. Here, we aimed at assessing whether LMV and SLM used in combination may act synergistically *in vitro* on inhibiting CMV replication.

Material and methods. The antiviral activity of LMV and SLM alone or in combination was evaluated by a checkerboard assay, using ARPE-19 cells infected with CMV strain BADrUL131-Y. LMV and SLM were used at concentrations ranging from 24 nM to 0.38 nM and 16 nM to 0.06 nM, respectively.

Results. The mean EC_{50} for LMV and SLM was 2.44 nM (95% CI, 1.66-3.60) and 1.40 nM (95% CI, 0.41-4.74), respective. LMV and SLM interaction yielded mainly additive effects over the range of concentrations tested.

Conclusion. The additive nature of the combination of LMV and SLM against CMV may have relevant clinical implications in management of CMV infection in allo-HSCT recipients undergoing prophylaxis with LMV.

Keywords: Cytomegalovirus; letermovir; sirolimus.

Evaluación *in vitro* del efecto combinado de letermovir y sirolimus en la replicación de citomegalovirus

RESUMEN

Introducción. Letermovir (LMV) se utiliza para la profilaxis de la reactivación de la infección y de la enfermedad orgánica por citomegalovirus (CMV) en adultos receptores de trasplante alogénico de células madre hematopoyéticas (alo-TPH) en pacientes seropositivos para CMV. A su vez, sirolimus (SLM), que muestra actividad anti-CMV *in vitro*, se usa con frecuencia para la profilaxis de la enfermedad de injerto contra huésped en alo-TPH. Nuestro objetivo fue evaluar si LMV y SLM utilizados en combinación pueden actuar sinérgicamente *in vitro* en inhibir la replicación del CMV.

Material y métodos. La actividad antiviral de LMV y SLM individualmente o en combinación se evaluó mediante un ensayo de tablero de ajedrez, utilizando células ARPE-19 infectadas con la cepa BADrUL131-Y de CMV. Se utilizaron LMV y SLM en concentraciones que variaron entre 24 nM y 0,38 nM y entre 16 nM y 0,06 nM, respectivamente.

Resultados. La EC_{50} media para LMV y SLM fue de 2,44 nM (IC del 95 %, 1,66-3,60) y 1,40 nM (IC del 95 %, 0,41-4,74), respectivamente. La interacción LMV y SLM produjo principalmente efectos aditivos en el rango de concentraciones ensayadas.

Conclusión. La naturaleza aditiva de la combinación de LMV y SLM frente a CMV puede tener implicaciones clínicas relevantes en el tratamiento de la infección por CMV en alo-TPH que reciben profilaxis con LMV.

Palabras clave: actividad in vitro; letermovir; sirolimus; citomegalovirus.

Correspondence:
David Navarro
Microbiology Service, Hospital Clínico Universitario, and Department of Microbiology, School of Medicine, Av. Blasco Ibáñez 17, 46010 Valencia, Spain.
E-mail: david.navarro@uv.es

INTRODUCTION

Cytomegalovirus (CMV) infection remains a significant cause of morbidity and mortality in the allogeneic hematopoietic stem cell transplantation (allo-HSCT) setting [1]. Although preemptive antiviral therapy (PET) has dramatically decreased the incidence of CMV end-organ disease [1], there is growing evidence that CMV DNAemia, especially if requiring PET, may be detrimental by increasing the risk of overall and non-relapse mortality [2-5]. Letermovir (LMV), an antiviral compound that blocks virion maturation by inhibiting the CMV DNA terminase complex [6], has been approved for prophylaxis of CMV reactivation and end-organ disease in adult CMV-seropositive allo-HSCT recipients. LMV efficiently suppresses CMV replication and seemingly increases survival by week 24 after allo-HSCT [7]. Nowadays, sirolimus (SLM), an mTOR inhibitor that inhibits T cell activation specifically by binding the cytosolic protein FKPB-12, is currently in wide use combined with other immunosuppressive agents for prophylaxis of acute graft-versus-host disease (aGvHD) in allo-HSCT. SLM exposure has been shown to dose-dependently decrease the risk of CMV DNAemia in a cohort of allo-HSCT recipients at high risk of CMV end-organ disease [8]. Furthermore, risk of CMV DNAemia requiring PET was shown to fall by 6% for each 1 ng/mL increase in SLM trough concentration [9]. The protective effect of SLM against CMV infection may be mechanistically related to the key role of the mTOR pathway in CMV protein synthesis and replication [10-14] and improvement of CMV-specific T-cell function via modulation of the environmental milieu [15]. The use of LMV and SLM in combination may improve the management of CMV infection in allo-HSCT by increasing clinical efficacy, reducing adverse effects and minimizing the likelihood of emergence of LMV-resistant variants. Evidence partly supporting the assumption that the association of LMV and SLM may reduce the rate of clinically significant CMV infection (CMV DNAemia that requires preemptive antiviral therapy/CMV end-organ disease) was recently provided [16]. A synergistic effect has been shown *in vitro* for maribavir, another anti-CMV drug inhibiting virus DNA replication, and SLM [17]. Here, we evaluated *in vitro* the potential interaction between SLM and LMV regarding its anti-CMV activity.

METHODS

Cells and virus. Human ARPE-19 retinal pigment epithelial cells (ATCC CRL-2302) were cultured in high-glucose Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (DMEM:F12K) (Gibco, ThermoFisher Scientific, Waltham, Massachusetts, USA), supplemented with 10% FCS (HyClone Laboratories Inc, Cytiva, USA), 10,000 IU penicillin (Gibco, ThermoFisher Scientific, Waltham, Massachusetts, USA), and 10 mg streptomycin (Gibco, ThermoFisher Scientific, Waltham, Massachusetts, USA). The CMV strain BADrUL131-Y4 [18] was kindly provided by Dr Pilar Pérez (ISCIII, Madrid, Spain). This strain is derived from a bacterial artificial chromosome clone of the CMV strain AD169 genome that was modified in *Escherichia coli* to express a functional UL131 protein, which permits replication in ARPE-19 and MRC-5 cells. Viral titers of CMV BADrUL131-Y4 were determined by limiting dilution in 96-well plates using MRC-5 cells.

Antiviral compounds. LMV was kindly provided by Merck, Sharp & Dohme (MSD) and stored as a stock solution of 50 milimolar (mM) in dimethyl sulfoxide (DMSO). A fresh working solution of 50 nanomolar (nM) was prepared in DMEM/F12K medium for each experiment and for medium renewal. SLM was purchased from Sigma Aldrich Merck (Merck KGaA, Darmstadt, Germany) and stored in a stock solution of 11 micromolar (µM) in DMSO. A working solution of 110 nM in in DMEM/F12K medium was prepared for each experiment.

Antiviral assay. The antiviral activity of LMV and SLM alone or in combination was evaluated by a checkerboard assay, as previously described [19]. ARPE-19 cells were seeded in 96-well microtiter plates ($4x10^4$ cells/well) for 24 h at 37ºC and 5% CO_2 and infected with 0.1 MOI of CMV strain BADrUL131-Y for 2 h in DMEM/F-12K medium containing either LMV (two-fold dilutions from 24 nM-14 ng/ml- to 0.38 nM- 0.22 ng/ml-), SLM (two-fold dilutions from 16 nM-15 ng/ml- to 0.06 nM-0.05 ng/ml-), or both drugs. The resulting two-dimensional matrix included all possible combinations of LMV and SLM and individual concentrations of each drug. The range of LMV and SLM concentrations tested in the current study was derived from that used in previous studies addressing the *in vitro* anti-CMV activity of LMV [6,17,20] or the potential synergistic effect between SLM and maribavir [17], respectively. For both drugs, these ranges were centered on the reported EC_{50} against CMV [6,17,20]. Four positive (CMV-infected cells in cell culture medium) and negative (CMV-uninfected cells in cell culture medium) controls were included in each plate. A total of five plates were prepared in three separate experiments. The plates were incubated for 6 days, and the medium (with or without the drugs) was replaced (with and without drugs, as appropriate for each well) every 2-3 days, as half-life for LMV and SLM are approximately of 12 and 60 hours, respectively, according to the corresponding manufacturer. After incubation, cells were washed, fixed with acetone/methanol (1:1), permeabilized with 0.2% Triton-X100 and stained for 1 h with 100 µl/well of a mouse IgG2a monoclonal antibody anti-Cytomegalovirus immediate early 1 (IE1) protein (1:150) (ThermoFisher, Waltham, Massachusetts, USA). A secondary FITC-labelled goat anti-mouse IgG2a cross-adsorbed secondary antibody (ThermoFisher, Waltham, Massachusetts, USA) was added at a dilution of 1:150 and incubated in dark for 1 h. Green-fluorescent nuclei were counted in a fluorescence microscope. Potential drug cytotoxicity was assessed for each experiment using mock-infected cells and matched drug exposure. Cell viability was verified after 6 days of culture for each condition using the alamarBlue cell viability assay (ThermoFisher, Waltham, Massachusetts, USA), following the manufacturer's recommendations.

Table 1 Analysis of the combination of letermovir and sirolimus by checkerboard matrix

Drug A	Max[a] (nM)	EC50[b] (95%CI)	Drug B	Max[c] (nM)	EC50[b] (95%CI)	Mean vol μM²%[d] (95%)		Mean vol μM²%[d] (99%)		Mean vol μM²%[d] (99.9%)	
						Synergy	Antagonism	Synergy	Antagonism	Synergy	Antagonism
Letermovir	24	2.44 (1.66-3.60)	Sirolimus	16	1.40 (0.41-4.74)	0	-172	0	-117	0	-76

95%CI, 95% Confidence Interval; EC50, 50% Effective Concentration; nM, nanoMolar; vol, volume.

[a]A total number of n=7 2-fold dilutions were tested starting from 24nM.

[b]The mean concentration capable of inhibiting Cytomegalovirus infection by 50% (EC50) was calculated by using the individual dose-response curve for each compound (Graphpad Prism software, v.6).

[c]A total number of n=9 2-fold dilutions were tested starting from 16nM.

[d]Volumes (μM²%) of synergy and antagonism were calculated using the MacSynergyII software. Values >50 μM2% were considered moderate and significant in vivo. Volumes at statistical level of confidence of 95%, 99% and 99.9% are shown.

Statistical analysis. EC_{50} was calculated by non-linear regression and using the dose-response curve obtained from the average percentage inhibition for each compound alone (GraphPad Prism software). Drug interaction was evaluated using MacSynergy II software, and interpreted as detailed elsewhere [20]. Differences between frequencies were analyzed using the Chi Square test. A *P* value <0.05 was considered statistically significant.

RESULTS

Both LMV and SLM inhibited CMV replication in ARPE-19 cells in a dose-dependent manner. The mean EC_{50} was 2.44 nM [1.40 ng/ml] (95% CI, 1.66-3.60) and 1.40 nM [1.28 ng/ml] (95% CI, 0.41-4.74), respectively (Table 1 and Figure 1 for dose-response curves). Overall synergy/antagonism volumes were 0% for synergy and -117% for antagonism at 99% confidence interval (Table 1). Interaction between the two drugs was concentration-dependent and mainly yielded additive effects (linear isobologram) over the range of concentrations tested (Figure 2), although low-level antagonistic interactions could be found, particularly at low concentrations of LMV (0.75 nM [0.43 ng/ml] and relatively high concentrations of SLM (8 nM [7.31 ng/ml]. Both LMV and SLM induced no cytotoxicity at the concentration ranges used during the incubation time, as revealed by the alamarBlue cell viability assay (not shown).

DISCUSSION

Prophylaxis with combined LMV and SLM is currently common practice in the allo-HSCT setting for prevention of CMV end-organ disease and aGvHD. Since both compounds display anti-CMV activity [6,10-12], we were interested in determining whether their interaction could result in a synergistic anti-CMV effect in epithelial ARPE-19 cells. To address this issue, we employed a conventional checkerboard assay using LMV and SLM concentrations centered around their EC_{50}

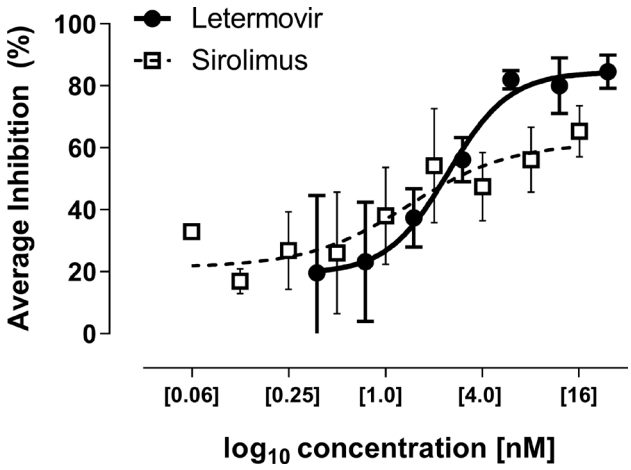

Figure 1 Dose-response curves of letermovir (LMV) and sirolimus (SLM) on CMV strain BADrUL131-Y infection of ARPE-19 cells. The percentage of inhibition represents the mean vaule of 5 experiments

against CMV [6,17,20] and SLM concentrations across the therapeutic range (4-12 ng/ml) [21]. Yet, due to the mechanism of action of both compounds and the choice of IE-1 as the assay read out full inhibition could not be achieved, as expected; in this sense, in the presence of LMV, CMV IE-1 can only be expressed in primarily infected cells (roughly 1/10 cells as an MOI of 0.1 was used); no further rounds of CMV replication ensue, as LMV blocks the generation of virus infectious particles. We obtained inhibition rates for LMV and SLM similar to previous studies [6,17,20], although our data indicated that the combination of LMV and SLM was additive at best. Notably, a low-level antagonistic effect could be observed in certain drug concentration combinations, in particular when

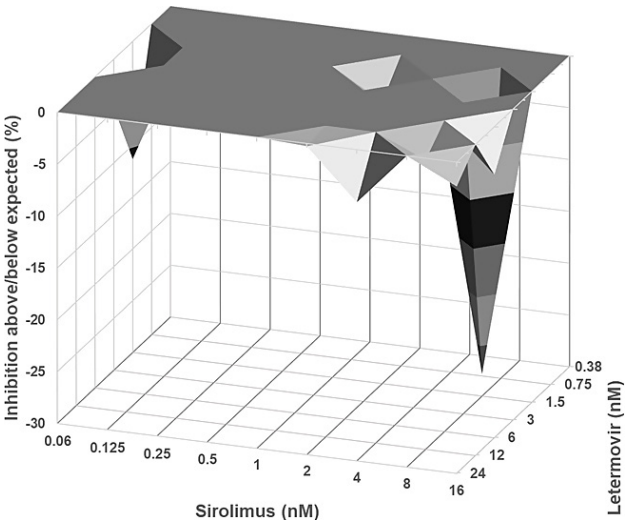

Figure 2 **Dimensional synergy plots for antiviral drug combinations with letermovir and sirolimus. The plots were generated from the results of 5 experiments of selected letermovir and sirolimus combinations against using MacSynergy II software. The difference between the observed viral growth inhibition and the amount expected from an additive effect of the drug concentrations was calculated at 99.9% confidence level.**

we used high concentrations of SLM and low concentrations of LMV. Although speculative, the lack of synergy between the two drugs and the antagonistic interactions documented could be mechanistically related to the ability of SLM to interfere with CAP-dependent translation by inhibiting mTOR kinase [4], which may impair expression of the LMV viral target, the CMV terminase complex. Whether our *in vitro* observations mirror *in vivo* interaction between these drugs is uncertain; in this context, two facts need to be considered; first, peak LMV levels reached during prophylaxis could be 100-fold higher (approximately 5**μ**M) than those used in our experiments [22], and thus CMV replication at mucosal and tissue sites could be drastically reduced *in vivo*, likely to a greater extent than in our *in vitro* model (around 50%); second, coadministration of LMV and SLM resulted in a 3.4-fold increases in area under the plasma concentration–time curve and 2.8-fold increases in maximum plasma concentration, respectively, of SLM [23].

The current study has several limitations. First, clinical CMV isolates were not used in the experiments; yet, given the nature of the recombinant CMV strain used herein (BAD-rUL131-Y4) [18], we do not foresee a straightforward biological reason that would account for LMV and SLM acting differently when employing clinical isolates. This assumption needs confirmation, though. Second, due to logistic reasons infectious virus yields could not be quantitated.

In summary, we have shown the additive nature of LMV and SLM *in vitro* interaction (over most drug concentrations assayed) in terms of anti-CMV activity in epithelial cells. Mechanistically the combination of LMV and SLM in vivo may thus shut off CMV replication *in vivo* at higher level than either drug used individually. Nevertheless, real-life data of the combined use of these drugs is required to gauge the potential clinical relevance of our *in vitro* observations.

ACKNOWLEDGEMENTS

Estela Giménez (Juan Rodés Contract, JR18/00053) and Eliseo Albert (Juan Rodés Contract; JR20/00011) hold contracts funded by the Health Institute Carlos III.

FUNDING

The present study was (partially) funded by Merck Investigator Study Program #58812. The funder had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

1. Ljungman P, de la Camara R, Robin C, Crocchiolo R, Einsele H, Hill JA, et al. Guidelines for the management of cytomegalovirus infection in patients with haematological malignancies and after stem cell transplantation from the 2017 European Conference on Infections in Leukaemia (ECIL 7). Lancet Infect Dis. 2019; 19(8):e260-e72. DOI: 10.1016/S1473-3099(19)30107-0.
2. Green ML, Leisenring W, Xie H, Mast TC, Cui Y, Sandmaier BM, et al. CMV viral load and mortality after hematopoietic cell transplantation: a cohort study in the era of preemptive therapy. Lancet Haematol.2016;3(3):e119-27. DOI: 10.1016/S2352-3026(15)00289-6.
3. Hill JA, Mayer BT, Xie H, Leisenring WM, Huang M-L, Stevens-Ayers T, et **al.** The cumulative burden of double-stranded DNA virus detection after allogeneic HCT is associated with increased mortality. Blood. 2017; 129(16):2316-25. DOI: 10.1182/blood-2016-10-748426 129:2316-2325.
4. Solano C, Vázquez L, Giménez E, de la Cámara R, Albert E, Rovira M, Espigado I, et al. Cytomegalovirus DNAemia and risk of mortality in allogeneic hematopoietic stem cell transplantation: Analysis from the Spanish Hematopoietic Transplantation and Cell Therapy Group. Am J Transplant. 2021;21(1):258-71. DOI: 10.1111/ajt.16147.
5. Giménez E, Torres I, Albert E, Piñana JL, Hernández-Boluda JC, Solano C, et al. Cytomegalovirus (CMV) infection and risk of mortality in allogeneic hematopoietic stem cell transplantation (Allo-HSCT): A systematic review, meta-analysis, and meta-regression

analysis. Am J Transplant. 2019;19(9):2479-2494. DOI: 10.1111/ajt.15515.

6. Goldner T, Hewlett G, Ettischer N, Ruebsamen-Schaeff H, Zimmermann H, Lischka P. The novel anticytomegalovirus compound AIC246 (Letermovir) inhibits human cytomegalovirus replication through a specific antiviral mechanism that involves the viral terminase. J Virol. 2011; 85(20):10884-93. DOI: 10.1128/JVI.05265-11.

7. Marty FM, Ljungman P, Chemaly RF, Maertens J, Dadwal SS, Duarte RF, et al. Letermovir prophylaxis for cytomegalovirus in hematopoietic-cell transplantation. New Engl J Med. 2017; 377(25):2433-44. DOI: 10.1056/NEJMoa1706640.

8. Piñana JL, Perez-Pitarch A, Guglieri-Lopez B, Giménez E, Hernandez-Boluda JC, Terol MJ, et al. Sirolimus exposure and the occurrence of cytomegalovirus DNA emia after allogeneic hematopoietic stem cell transplantation. Am J Transplant. 2018; 18(12):2885-94. DOI: 10.1111/ajt.14754.

9. Guglieri-Lopez B, Perez-Pitarch A, Garcia-cadenas I, Gimenez E, Barba P, Rabella N, et al. Effect of sirolimus exposure on the need for pre-emptive antiviral therapy for CMV infection after allogeneic hematopoietic stem cell transplantation. Biol Blood Marrow Transplant. 2019; 25(5):1022-30. DOI: 10.1016/j.bbmt.2019.01.012.25:1022-1030.

10. Kudchodkar SB, Yu Y, Maguire TG, Alwine JC. Human cytomegalovirus infection induces rapamycin-insensitive phosphorylation of downstream effectors of mTOR kinase. J Virol. 2004; 78(20):11030-9. DOI: 10.1128/JVI.78.20.11030-11039.2004.

11. Nashan B, Gaston R, Emery V, Säemann MD, Mueller NJ, Couzi L, et al. Review of cytomegalovirus infection findings with mammalian target of rapamycin inhibitor-based immunosuppressive therapy in de novo renal transplant recipients. Transplantation. 2012; 93(11):1075-85. DOI: 10.1097/TP.0b013e31824810e6.

12. Kudchodkar SB, Yu Y, Maguire TG, Alwine JC. Human cytomegalovirus infection alters the substrate specificities and rapamycin sensitivities of raptor-and rictor-containing complexes. Proc Natl Acad Sci. 2006; 103(38):14182-7. DOI: 10.1073/pnas.0605825103.

13. Poglitsch M, Weichhart T, Hecking M, Werzowa J, Katholnig K, Antlanger M, et al. CMV late phase-induced mTOR activation is essential for efficient virus replication in polarized human macrophages. Am J Transplant. 2012; 12(6):1458-68. DOI: 10.1111/j.1600-6143.2012.04002.x.

14. Moorman NJ, Shenk T. Rapamycin-resistant mTORC1 kinase activity is required for herpesvirus replication. J Virol. 2010; 84(10):5260-9. DOI: 10.1128/JVI.02733-09.

15. Bak S, Tischer S, Dragon A, Ravens S, Pape L, Koenecke C, et al. Selective effects of mTOR inhibitor sirolimus on naive and CMV-specific T cells extending its applicable range beyond immunosuppression. Front Immunol. 2018; 9:2953. DOI: 10.3389/fimmu.2018.02953.

16. Xue E, Lorentino F, Clerici D, Farina F, Oltolini C, Giglio F, et al. Coadministration of letermovir and sirolimus in allogeneic hematopoietic cell transplant recipients. Bone Marrow Transplant. 2022;57(1):128-130. DOI: 10.1038/s41409-021-01486-5.

17. Chou S, Ercolani RJ, Derakhchan K. Antiviral activity of maribavir in combination with other drugs active against human cytomegalovirus. Antiviral Res. 2018; 157:128-33. DOI: 10.1016/j.antiviral.2018.07.013.

18. Wang D, Shenk T. Human cytomegalovirus UL131 open reading frame is required for epithelial cell tropism. J Virol. 2005; 79(16):10330-8. DOI: 10.1128/JVI.79.16.10330-10338.2005.

19. Prichard MN, Shipman Jr C. A three-dimensional model to analyze drug-drug interactions. Antiviral Res. 1990; 14(4-5):181-205. DOI: 10.1016/0166-3542(90)90001-n.

20. Wildum S, Zimmermann H, Lischka P. In vitro drug combination studies of letermovir (AIC246, MK-8228) with approved anti Human Cytomegalovirus (HCMV) and HIV compounds in inhibition of HCMV and HIV replication. Antimicrob Agents Chemother. 2015; 59(6):3140-8. DOI: 10.1128/AAC.00114-15.

21. Stenton SB, Partovi N, Ensom MH. Sirolimus: the evidence for clinical pharmacokinetic monitoring. Clin Pharmacokinet. 2005;44(8):769-86. doi: 10.2165/00003088-200544080-00001.

22. Isberner N, Aghai F, Schirmer D, Kraus S, Grigoleit GU, Klinker H. Quantification of letermovir in human serum using high-performance liquid chromatography with diode array detection. J Chromatogr B Analyt Technol Biomed Life Sci. 2020; 1159:122399. DOI: 10.1016/j.jchromb.2020.122399.

23. McCrea JB, Macha S, Adedoyin A, Marshall W, Menzel K, Cho CR, et al. Pharmacokinetic Drug-Drug Interactions Between Letermovir and the Immunosuppressants Cyclosporine, Tacrolimus, Sirolimus, and Mycophenolate Mofetil. J Clin Pharmacol. 2019;59(10):1331-1339. doi: 10.1002/jcph.1423.