The *in vitro* antiviral activity of the anti-hepatitis C virus drugs daclatasvir and sofosbuvir against SARS-CoV-2

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Background

The SARS-CoV-2 pandemic continues to cause major global morbidity, mortality and economic burden. The public health urgency led to the study of clinically approved drugs as repurposed medicines to treat individuals with COVID-19. The current approaches have not proven successful to date. The understanding of preclinical activity, mechanism of action, pharmacokinetics and safety are critical to achieving clinical benefits from repurposing drugs.

Direct-acting antivirals (DDA) against hepatitis C virus (HCV) are among the safest antiviral agents, since they become routinely used in the last five years. Due to their recent incorporation among therapeutic agents, drugs like daclatasvir (DCV) and sofosbuvir (SFV) have not been systematically tested against SARS-CoV or MERS-CoV. DCV inhibits HCV replication by binding to the N-terminus of non-structural protein (NS5A), affecting both viral RNA replication and virion assembly. NS5A is a multifunctional protein in the HCV replicative cycle, involved with recruitment of host cellular lipid droplets, RNA binding and replication, protein-phosphorylation, cell signaling and antagonism of interferon pathways. In large positive sense RNA viruses, such as SARS-CoV-2, these activities are executed by various viral proteins, especially the non-structural proteins (nsp) 1 to 14. SFV inhibits the HCV protein NS5B, its RNA polymerase. The similarities between the SARS-CoV-2 and HCV RNA polymerase provide a rational for studying sofosbuvir as an antiviral for COVID-19.

Taken collectively, current data provided a bases to investigate whether DCV and SFV could inhibit the production of infectious SARS-CoV-2 particles in physiologically relevant cells.

Results

DCV is more potent than SFV to inhibit the production of infectious SARS-CoV-2 particles

Resumed Methodology: Inhibition assays were performed at MOI of 0.01 for Vero cells 24h after infection, and 0.1 for HuH-7 and Calu-3 cells at 48h after infection. Cultures were treated after 1h infection period and cell culture supernatant fractions were harvested to measure infectious SARS-CoV-2 by plaque forming units (PFUs) in Vero cells. Cytotoxicity assays were performed in Vero, Huh-7 and Calu-3 through XTT reduction assays.

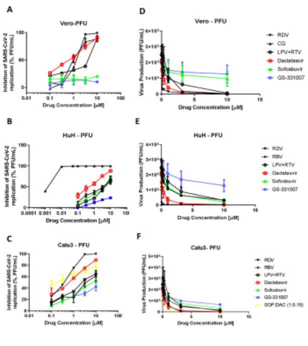


Figure 1. **The antiviral activity of daclatasvir (DCV) and sofosbuvir (SFV) against SARS-CoV-2.** Vero (A), HuH-7 (B) or Calu-3 (C and D) cells were infected with SARS-CoV-2, for 1h at 37 °C. Inoculum was removed, cells were washed and incubated with fresh DMEM containing 2% fetal bovine serum (FBS) and the indicated concentrations of the DCV, SFV, chloroquine (CQ), lopinavir/ritonavir (LPV+RTV) or ribavirin (RBV). Viral replication in the culture supernatant was measured by PFU/mL. Results are displayed at percentage of inhibition (A-C) or virus titers (D). The data represent means ± SEM of three independent experiments.

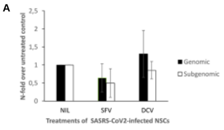
Table 1 – The pharmacological parameters of SARS-CoV-2 infected cell in the presence of DCV and SFV

	Vero			Huh-7			Calu-3		
Drugs	EC50	CC50	SI	EC50	CC50	SI	EC50	CC50	SI
DCV	0.8 ± 0.3	31 ± 8	39	0.6 ± 0.2	28 ± 5	47	1.1 ± 0.3	38 ± 5	34
SFV	>10	360 ± 43	ND	5.1 ± 0.8	381 ± 34	74	7.3 ± 0.5	512 ± 34	70
GS-331007	>10	512± 24	ND	>10	421 ± 18	ND	9.3 ± 0.2	630 ± 34	68
DCV/SFV	ND	ND	ND	ND	ND	ND	0.7 ± 0.2	389 ± 12	555
RBV	ND	ND	ND	6.5 ± 1.3	142 ± 12	13	7.1 ± 0.5	160	16
CQ	1.3 ± 0.4	268 ± 23	206	ND	ND	ND	ND	ND	ND
LPV/RTV	5.3 ± 0.5	291 ± 32	54	2.9 ± 0.2	328 ± 16	113	8.2 ± 0.3	256 ± 17	31

EC_{50}, and CC_{50} are described in µM

Protective effect of SFV and DCV in non-permissive cells

Resumed Methodology: Neurons and monocytes do not present productive replication of SARS-CoV-2, however, infection of these cells is known to be associated with neuro-COVID-19 and cytokine storm, respectively. Therefore, these cell were used in inhibition assays. Neural Stem Cells (NSCs), NSCs-based neurospheres, and human primary monocytes were infected at MOI 0.1 for 2h at 37 °C, inoculum was removed and fresh medium containing the compounds was added. After 24h (monocytes) and 5 days (neurospheres), cell death was measured by TUNEL approach, virus levels in the supernatant were quantified by RT-PCR and cytokines measured by ELISA.



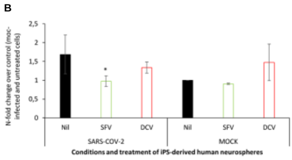


Figure 2. **Sofosbuvir (SFV) inhibits SARS-CoV-2 replication in human iPS cell-derived NSCs.** (A) NSCs were infected at MOIs of 0.1 and treated with 1 µM of SFV or daclatasvir (DCV). After 5 days, the culture supernatants were collected, and the virus was quantified by RNA levels using RT-PCR. (B) NSCs in spheroid format were labeled for Tunel and DAPI after 5 days post-infection. The data represent means ± SEM of three independent experiments. * P < 0.05 for the comparison between the SARS-CoV-2-infected cells untreated (nil) vs treated with SFV..

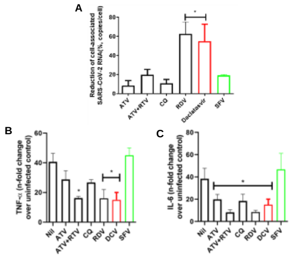


Figure 3. **Daclatasvir (DCV) impairs SARS-CoV-2 replication and cytokine storm in human primary monocytes**. Human primary monocytes were infected at the MOI of 0.01 and treated with 1 µM of daclatasvir (DCV) sofosbuvir (SFV), chloroquine (CQ), atazanavir (ATV) or atazanavir/ritonavir (ATV+RTV). After 24h, cell-associated virus RNA loads (A), as well as TNF-α (B) and IL-6 (C) levels in the culture supernatant were measured. The data represent means ± SEM of experiments with cells from at least three healthy donors. * P < 0.05 comparison between untreated cells (nil) and each specific treatment.

DCV and SFV may target different events during SARS-CoV-2 RNA synthesis

Resumed Methodology: Time-of-addition assay was performed to gain insights on the temporality of DCV`s activity against SARS-CoV-2. Vero cells were infected at MOI of 0.01 and treated at different time points, with DCV at 2-fold its EC_{50}. Viruses present in the supernatants were titrated by PFU/mL. To confirm the rational that both SFV and DCV inhibit viral RNA synthesis, intracellular levels of SARS-CoV-2 genomic and sub genomic RNA were measured in Calu-3 cells through real time RT-PCR. Molecular docking methods were applied to predict the complexes with lowest energy interactions between the SARS-CoV-2 RNA polymerase and the active metabolite of SFV as well as DCV.

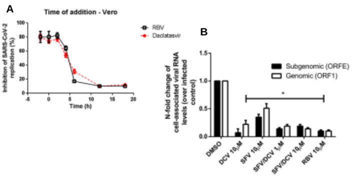


Figure 4. **Daclatasvir (DCV) and sofosbuvir (SFV) reduced SARS-CoV-2 associated RNA synthesis.** (A) To initially understand the temporal pattern of inhibition promoted daclatasvir, we performed by Time-of-addition assays. Vero cells were infected with MOI 0f 0.01 of SARS-CoV-2 and treated with DCV or ribavirin (RBV) with two-times their EC_{50} values at different times after infection, as indicated. After 24h post infection, culture supernatant was harvested and SARS-CoV-2 replication measured by plaque assay. (B) Next, Calu-3 cells were infected with SARS-CoV-2 at MOI of 0.1, for 1h at 37 °C. Inoculum was removed and cells were incubated with the indicated concentrations of the DCV, SFV or RBV at 10 µM. After 48h, cells monolayers were lysed, total RNA extracted and quantitative RT-PCR performed for detection of ORF1 and ORFE mRNA. The data represent means ± SEM of three independent experiments. * P< 0.05 for comparisons with vehicle (DMSO). # P< 0.05 for differences in genomic and sub-genomic RNAs.

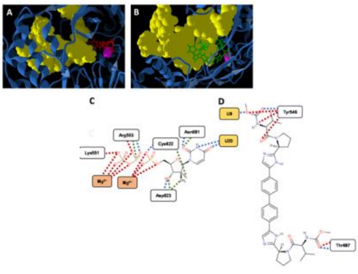


Figure 5. *(A) Cartoon representation of SARS-Cov-2 RNA polymerase (nsp12; blue) with RNA template (yellow), and Mg2+ (pink) ions, in CPK representation, complexed to the active metabolite of sofosbuvir (SFV; red) (A) and daclatasvir (B). Schematic representations of the hydrogen bonds (H-bonds; blue dashed lines), attractive electrostatic interactions (red dashed lines), and steric interactions (green dashed lines) present in the nsp12-SFV (C) and nsp12-daclatasvir (D) complexes. The nsp12 residues, RNA nucleotides, and Mg2+ ions are represented by white, yellow, and orange rectangles.*

DCV effect on SARS-CoV-2 RNA

Resumed Methodology: Molecular modeling predictions and melting curves of extracted viral RNA was generated to assess whether DCV could affect the virus RNA folding. The thermal melting profiles of the RNA and RNA/DCV complexes were obtained by varying the temperature in a regular real time thermocycler. Continuous passages of SARS-CoV-2 in the presence of DCV were performed in order to evaluated the generation of mutations in viral RNA that may result in a change of its secondary structure. Viruses were passaged in Vero cells at the MOI of 0.1 during two months in the presence of increasing concentrations of DCV (up to 7 µM). The virus RNA was submitted to unbiased sequence using a MGI-2000 and a metatranscriptomics approach.

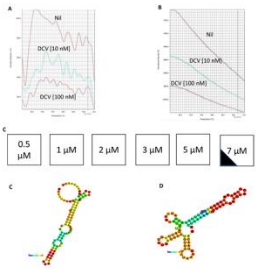


Figure 6. **Daclatasvir (DCV) favors SARS-CoV-2 RNA unfold.** A total of 10 ng of SARS-CoV-2 RNA was incubated with 10 or 100 nM of DCV during a standard melting curve in the presence of picogreen, derivative (A) and normalized (B) reports are presented. (C) the scheme represent the percentage of wild-type (WT; white) and mutant (black) virus after growing SARS-CoV-2 in Vero Cells at a MOI 10 times higher than used in other experiments, 0.1, and sequentially treated with sub-optimal doses of DCV. Each passage was done after 2-4 days pos-infection, when cytopathic effect was evident. Virus RNA was unbiased sequenced using a MGI-2000 and a metatrasncriptomic approach was employed during the analysis. WT (D) and mutant (E) SARS-CoV-2 secondary RNA structure encompassing the nucleotides 28169-28259 are presented.

Table 2 – Genetic and biochemical characteristics of the DCV- mutant SARS-CoV-2.

Type	Sequences	Secondary structure	Thermodynamic ensemble (Kcal/mol)	Identity to SARS-CoV-2 genomes
Wild-Type	TTTTTAGAGTATCATGACGTTCGTGTT GTTTTAGATTTCATCT AAACGAACAAACTAAAATGTCTGATA ATGGACCCCAAAATCAGCG	[illegible]	-17.67	99%
Mutant	TTTTTAGAGTATCATGACTTTCGATCTC TTGTAGATCTGTTCTCT AAACGAACAAACTAAAATGTCTGATA ATGGACCCCAAAATCAGCG	[illegible]	-14.21	89%

Mutations in red, Insertions in bold

Physiologically based pharmacokinetic (PBPK) modeling for DCV

Resumed Methodology: PBPK model was constructed in Python 3.5 and simulated using a population of one hundred virtual healthy individuals (50% female) between 20-60 years and having weight and height as provided by the US national health statistics reports. A seven compartmental absorption and transit model representing the various parts of the duodenum, jejunum and ileum to capture effective absorption kinetics was used in the model. DCV PBPK model was validated in healthy individuals using available data in humans for various single doses – 1, 10, 25, 50, 100 and 200 mg and for various multiple doses – 1, 10, 30 and 60 mg at fasted state. For the inhibition of SARS-CoV-2, a mean target concentration (EC_{90}) of 4.12 µM or 3079 ng/ml obtained from multiple in vitro studies was used

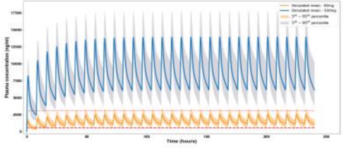


Figure 7. **Predicted daclatasvir plasma concentration for multiple 60 mg and 330 mg TID doses.** The dotted and the dashed lines represent the EC90 and EC50 values of daclatasvir for SARS-CoV-2

Conclusions

- Altogether, our data reveal that SFV and DCV inhibited SARS-CoV-2 replication in physiologically relevant cells, including type II pneumocytes. Besides, the drugs prevented virus-induced neuronal apoptosis and release of cytokine storm-related inflammatory mediators by monocytes, respectively. Both drugs inhibited independent events during RNA synthesis and this was particularly the case for DCV, which also targeted secondary RNA structures in the SARS-CoV-2 genome.
- In summary, effective early antiviral interventions are urgently required for the SARS-CoV-2 pandemic to improve patient clinical outcomes and disrupt transmission at population level. The presented data for two widely available anti-HCV drugs, particularly for DCV, provide a rational basis for further validation of these molecules for anti-SARS-CoV-2 interventions.

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