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Complexation of a 1-Indanone Thiosemicarbazone with Hydroxypropyl-β-Cyclodextrin Enhances Its Activity Against a Hepatitis C Virus Surrogate Model

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The current standard of care of the infection by hepatitis C virus (HCV) is effective in a limited number of patients and the high cost hinders therapy affordability and compliance. In this context, the research of new direct-acting antiviral agents (DAAs) for a more effective and long-lasting therapy is an urgent need and an area of active investigation. In an effort to develop novel DAAs, a series of 1-indanone thiosemicarbazones (TSCs) was synthesized and fully characterized. However, the high self-aggregation tendency and extremely poor aqueous solubility of these antiviral candidates often preclude their reliable biological evaluation *in vitro*. To maintain constant TSC concentrations over the biological assays, different TSC/cyclodextrin complexes were produced. In the present work, we report for the first time the cytotoxicity and antiviral activity of 5,6-dimethoxy TSC inclusion complexes with hydroxypropyl-β-cyclodextrin on bovine viral diarrhea virus (BVDV) as HCV surrogate model. Results showed a potent suppression of the virus replication, with greater activity for the inclusion complexes than the free compound.

Keywords: Bovine Viral Diarrhea Virus (BVDV), HCV Surrogate Model, 1-Indanone Thiosemicarbazones, Hydroxypropyl-β-Cyclodextrin Inclusion Complexes, Direct-Acting Antiviral Agents.

1. INTRODUCTION

The hepatitis C virus (HCV) is the etiologic agent of the most widespread infectious disease in the world and plays a key role in the development of hepatocellular carcinoma (HCC).[1–3] One of the main features of this virus is its high rate of progression to chronicity that implies a chronic persistent infection in 70–85% of the patients, where 25% will develop HCC after 20–30 years of the primary infection.[2, 3] Moreover, this is an asymptomatic disease for a variable period of time, so the number of infected people is unknown.[2, 3] The genetic heterogeneity generated during the virus replication resulted in at least six major HCV genotypes and more than eighty subtypes which have multiple evolutionary characteristics in terms of both the pathogenesis and the response to the antiviral therapy.[4] The geographical distribution of these genotypes and subtypes varies significantly. HCV genotypes 1a and 1b represent 60% worldwide and this is associated with a lower rate of response to treatment.[2–4] In addition, significant genetic variability hinders the development of a HCV vaccine. The current standard of care is the combination

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of ribavirin (RBV), a synthetic nucleoside,[5] with the classic immunomodulator interferon (IFN) or PEGylated-IFN-alpha (PEG-IFN-α).[6] It has been shown that a prolonged treatment with RBV/IFN leads to a transitory virologic response. However, this therapy is usually associated with severe hematological side effects and significant costs that constrains the access to a minority of patients.[7, 8] Furthermore, NS3 serine protease inhibitors recently introduced into the market must be administered in combination with RBV/IFN.[9] The absence of robust *in vitro* and *in vivo* models that maintain replication of the virus and, therefore, allow the study of infection mechanisms, pathogenicity and therapeutic strategies, has become a major hurdle to achieve noteworthy progresses in the prophylaxis and treatment of the infection.[10, 11]

Bovine viral diarrhea virus (BVDV) is an easy cultivable virus and one of the best characterized members of the *Pestivirus* genus. This genus and the genera *Flavivirus* and *Hepacivirus* constitute the family *Flaviviridae*. The genomic organization, translation, replication pathway and protein functions of pestiviruses closely resemble those of HCV. In this context, BVDV is widely used as surrogate HCV model for

(i) the evaluation of novel compounds with potential anti-HCV activity and

(ii) the molecular study of viral protein[12–14]

In addition, available full-length (FL) and sub-genomic (SG) HCV replicon systems represent a great alternative for the advancement of the study of the viral cycle, the resistance mechanisms to various existing therapies, the development of potential vaccines and the research and optimization of DAAs.[15–17] In certain aspects of viral replication, BVDV is a more advantageous model than the current HCV replicon system.[18] In this context, Castro et al. have reported on the anti-BVDV activity of different 1-indanone TSCs and established the synergism between 5,6-dimethoxy TSC (Fig. 1(A)) and RBV as a potent novel non-nucleoside inhibitor (NNI) of the BVDV RNA-dependent RNA polymerase (RdRp).[18–20] Furthermore, this TSC displayed a seven times higher selectivity index (SI = 80.29) than RBV (SI = 11.64).[18] It is important to mention that the introduction of the two methoxy functional groups in positions 5 and 6 of the TSC aromatic ring (Fig. 1(A)) was identified as a key structural feature that confers highly selective and potent inhibitory activity of the BVDV replication cycle.[18] In a different study, the antiviral activity of this TSC was assessed on FL and SG subgenotype 1b HCV replicon systems;[21] the mechanism most likely involves the inhibition of non-structural (NS) proteins. Nevertheless, the high self-aggregation tendency and the extremely poor aqueous solubility of these antiviral candidates remain a hurdle towards their reliable biological evaluation *in vitro*.[22] To overcome this, the production of inclusion complexes of TSC with different cyclodextrins was comprehensively studied;[23] cyclodextrins are cyclic oligosaccharides that display a hydrophobic cavity that enables the incorporation of lipophilic molecules (Fig. 1(B)).[24] In addition, TSC/hydroxypropyl-β-cyclodextrin (HPβ-CD) complexes suppressed the HCV replication in FL and SG replicon systems.[21] Since HPβ-CD is cited in the list of Inactive Pharmaceutical Ingredients of FDA, it has attracted the attention of pharmaceutical scientists for the solubilization and delivery of hydrophobic investigational drugs *in vitro* and in preclinical and clinical assays.[24] To gain a deeper understanding of the potential of this technology platform and expand its applicability to overcome the main biopharmaceutic drawback of TSCs, in this work, we assessed for the first time the cytotoxicity and antiviral activity of TSC/HPβ-CD complexes on BVDV.

Based on our previous results,[21] two TSC/HPβCD complexes (complex A: 22 μM/0.50% w/v; complex B: 22 μM/0.25% w/v) were prepared in sterile water and diluted in Infection Medium (IM; Minimal Essential Medium supplemented with Donor Horse Serum 5%) to render two stock solutions with TSC/HPβCD concentrations of 11 μM/0.25% w/v and 11 μM/0.125% w/v that were further diluted (Table I). The preparation of TSC/CD complexes was carried out by the co-solvent method, in duplicate. For this purpose, 5,6-dimethoxy-1-indanone TSC (Fig. 1(A)) was firstly synthetized and purified as depicted elsewhere.[18] 2-Hydroxypropyl beta-CD (Fig. 1(B); Cavasol® W_7 HP; HPβ-CD; molar substitution, MS, per anhydro glucose unit of 0.65; average molecular weight, M_W, of 1400 g/mol, Wacker-Chemie GmbH Germany) was dissolved in methanol and TSC

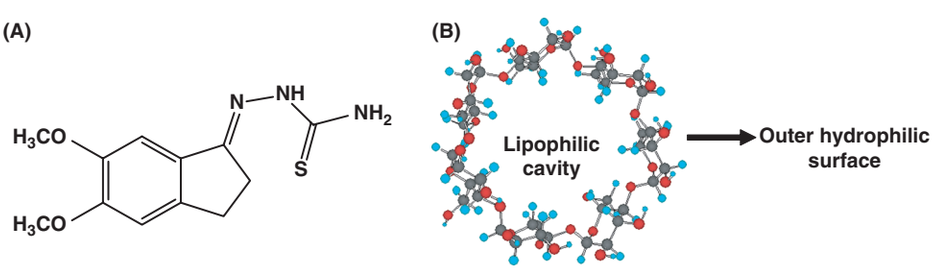

Figure 1. Chemical structure of (A) 5,6-dimethoxy-1-indanone TSC and representative structure of (B) 2-hydroxypropyl-β-cyclodextrin included in the cytotoxicity and anti-BVDV assays.

Table I. Final concentrations of HPβ-CD and TSC in TSC/HPβ-CD A and B inclusion complexes used in both cytotoxicity and anti-BVDV activity assays.

HPβ-CD (% w/v)		
TSC/HPβ-CD A	TSC/HPβ-CD B	TSC (μM)
0.250	0.125	11.0
0.125	0.063	5.5
0.063	0.031	2.75
0.031	0.016	1.38
0.016	0.008	0.69

(in slight excess) was dissolved in chloroform:acetone (1:1). Then, both solutions were thoroughly mixed over 15 min under magnetic stirring, at 25 °C. The solvent was removed under vacuum using a rotary evaporator (15 min, 70–90 °C, Fbr®, Decalab S.R.L, Argentina). White powders were re-dissolved in MilliQ water, the volume being in accordance with the final cyclodextrin desired concentrations: 0.25% and 0.50% w/v, both containing 22 μM o f TSC. Solutions were magnetically stirred (30 min, 25 °C) to solubilize the complex and finally filtered (0.45 μm, GE nitrocellulose mixed esters membrane plastic syringe filters). The presence of organic solvent residues could lead to cytotoxic effects during the *in vitro* biological evaluation of the TSCs in cell monolayers. To ensure the complete elimination of the solvents employed during the preparation of the complexes, samples were analyzed by gas chromatography. The concentration of residual MeOH (the solvent with the higher boiling point of the mixtures used) was smaller than 100 ppm. The maximum concentration allowed by the European Pharmacopeia is 3000 ppm. Finally, 1:2, 1:4, 1:8 and 1:16 dilutions were performed using infection medium (IM) 2*X* (Table I). Then, the cytotoxicity and the anti-BVDV (type-1 NADL strain) activity of HPβ-CD, free TSC and TSC/HPβ-CD complexes were assayed in a Madin-Darby bovine kidney (MDBK) cell line by means of the 3-(4,5-dimethylthiazol- 2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)/phenazine methosulfate (MTS/PMS, Promega) method and the reduction of the viral cytopathic effect (CPE), respectively.[18–20]

For cytotoxicity studies Madin-Darby bovine kidney (MDBK) cells were seeded in 96-well plates (1×10^4 cells per well) and maintained with Minimal Essential Medium (MEM, Gibco) supplemented with Fetal Bovine Serum (10%) for 24 h (37 °C, 5% CO_2). Then, serial dilutions of free TSC and TSC/HPβ-CD complexes in MEM supplemented with 2.5% Donor Horse serum (DHS) were added to each well and cells were allowed to proliferate for 3 days at 37 °C, after which the cell number was determined by means of MTS/PMS method. The formazan product formed is proportional to the number of living cells. After 3 h at 37 °C, the absorbance was determined at 490 nm. The percentage of cell viability was calculated as follows

$$\text{Cell viability} = Abs_t \times 100/Abs_c$$

Where, Abs_c is the absorbance of untreated cells (control) and Abs_t, the absorbance of cells treated with a certain TSC dilution.

For the assessment of the anti-BVDV activity, MDBK cells were seeded in in 96-well plates (1×10^4 cells per well) and maintained as described above for the assessment of the cytotoxicity for 24 h. Then, serial dilutions of free TSC and TSC/HPβ-CD complexes were added to each well and the BVDV inoculum added (multiplicity of infection, MOI of 0.01). This inoculum resulted in a cytophatic effect (CPE) greater than 80% after 3 days of incubation at 37 °C. Mock infected (MI) and infected MDBK cells without TSC (IC) were included as controls in each assay. After 3 days, the medium was removed and MEM-DHS supplemented with MTS/PMS solution was added to each well. The absorbance of each well was read at 490 nm. Dose-response curves were drawn by calculating the % of viral CPE inhibition for each concentration of compound, were Abs_{MI}–Abs_{IC} was considered as 100%. The 50% effective concentration (EC_{50}) was defined as the TSC concentration that resulted in 50% protection of the cells against virus-induced CPE and was calculated using interpolation from dose-response curves adjusted by a simple linear regression. The results were obtained from at least two independent assays, in which concentration of compound was tested at least in quadruplicate, and analysed by Student *t* test using InfoStat software version 2011 (Di Rienzo J. A., F. Casanoves, M. G. Balzarini, L. Gonzalez, M. Tablada, and C. W. Robledo. InfoStat version 2011. Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina. URL http://www.infostat.com.ar).

The results obtained in the present work shown that HPβ-CD was not cytotoxic up to a concentration of 0.25% w/v, viability levels being 88.7% and 91.8% for 0.125% and 0.25% w/v cyclodextrin samples, respectively (Fig. 2(A)). Conversely, 0.5% and 1% w/v samples led to a statistically significant decrease of cell viability with respect to untreated cell controls ($P < 0.05$). It is worth stressing that the intrinsic cytotoxicity of cyclodextrins is a well established phenomenon attributed to the interaction of some derivatives, especially β-cyclodextrins, with the cellular membrane and depends on both the concentration and the time of exposure.[25] As expected, these non-cytotoxic concentrations resulted inactive against BVDV (Fig. 2(A)). Therefore, having ruling out any possible interference in the antiviral assays, two TSC/HPβ-CD complexes with initial cyclodextrins concentrations that were not cytotoxic and their 1:2, 1:4, 1:8 and 1:16 dilutions in IM were assessed. The final TSC and HPβ-CD concentrations in the complexes are summarized in Table I. Both TSC/HPβ-CD complexes were not cytotoxic in MDBK

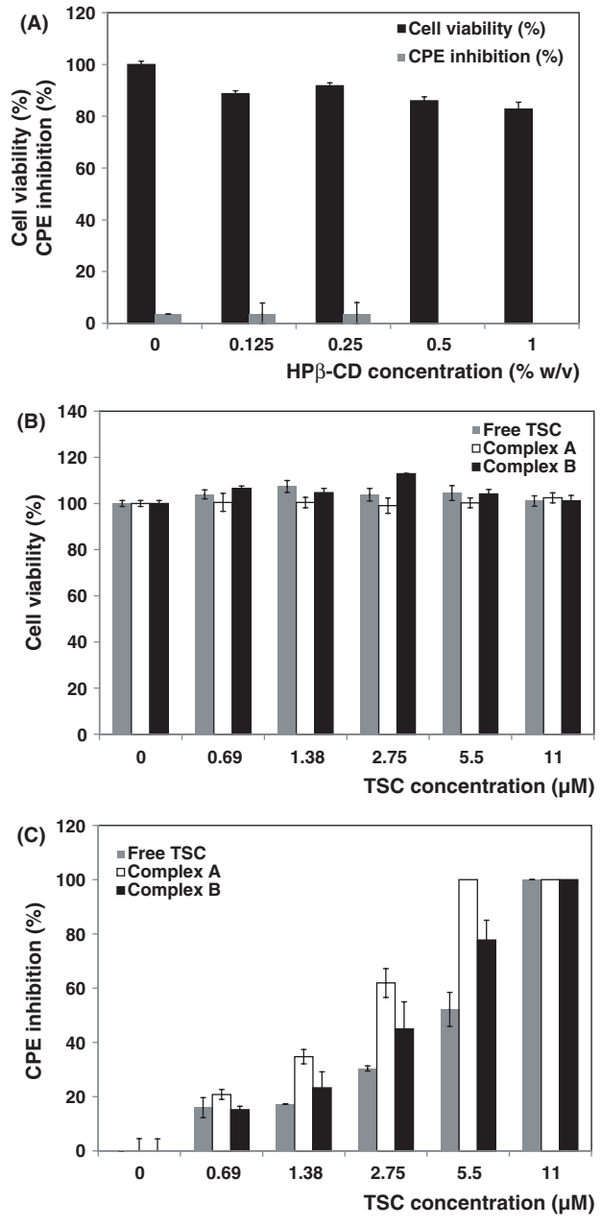

Figure 2. Biological evaluation of HPβ-CD, free TSC and TSC/HPβ-CD complexes in MDBK cells. (A) Cytotoxicity and anti-BVDV activity of HPβ-CD, (B) cytotoxicity of all the samples and (C) anti-BVDV activ-ity of all the samples. The cytotoxicity was evaluated by the MTS assay and the anti-BVDV activity by viral cytopathic effect (CPE) reduction method and expressed as % of CPE inhibition. Mock infected cells were included as controls. The results were obtained from at least two inde-pendent assays and four replicates and they are expressed as mean±S.D.

cells (Fig. 2(B)), these data being consistent with those obtained in FL and SG replicon systems.[21] When the anti-BVDV activity was evaluated, the inclusion complexes showed a gradual improvement of the antiviral potency and, consequently, lower effective concentration 50 values (EC_{50}; defined as the TSC concentration that reduces by 50% viral CPE, Table II) than free TSC (Fig. 2(C)).

Table II. Anti-BVDV activity of TSC and TSC/HPβ-CD A and B inclusion complexes, expressed as EC_{50}. Results are expressed as the mean value of two independent assays ± S.D. Each assay is the results of at least four replicates.

Sample	EC_{50}^{b} (μM) (± S.D.)
Free TSC[a]	5.35 (0.57)
Complex A	2.32 (0.09)
Complex B	3.42 (0.47)

Notes: [a]Free TSC was solubilized in DMSO and the final concentration of the solvent in the culture medium was below 1% v/v; [b]Fifty percent effective concentration (EC_{50}) was defined as the concentration of TSC that offered 50% protection of the cells against the virus-induced cytopathic effect (CPE) and was calculated by interpolation from dose-response curves adjusted by lineal regression (see supporting information).

For example, free TSC 5.5 μM resulted in 52.1% of CPE inhibition, while this value increased to 77.8% and 100% for complexes B and A, respectively (Fig. 2(C)). The most pronounced increase in the inhibition curves was provided by complex A (Fig. 2(C)); this complex contains a higher cyclodextrin concentration than complex B. This is understandable in view of the greater concentration of HPβ-CD in this complex that lowered the effective concentration of free TSC available to exert the antiviral activity. These results stress the relevance of fine tuning the concentration of the drug carrier to improve the physical stability of the investigational drug in solution, while enabling its release during the biological assay.

One of the most remarkable hurdles toward the clinical implementation of a new drug entity stems from the poor water solubility, precluding not only preclinical and clinical stages but also early *in vitro* biological assays and contributing to increase the drug attrition rates. The results presented here constitute further evidence that HPβ-CD not only improves the solubility of the poorly water-soluble compound under investigation but it also improves its antiviral potency *in vitro* without cytotoxicity or any direct antiviral activity. In more advanced studies, this platform could be exploited for the study of the pharmacokinetics and toxicology of this inhibitor in animal models.

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