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Antiviral activity against the hepatitis C virus (HCV) of 1-indanone thiosemicarbazones and their inclusion complexes with hydroxypropyl-β-cyclodextrin

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ABSTRACT

The hepatitis C virus (HCV) is a major cause of acute and chronic hepatitis in humans. Approximately 5% of the infected people die from cirrhosis or hepatocellular carcinoma. The current standard therapy comprises a combination of pegylated-interferon alpha and ribavirin. Due to the relatively low effectiveness, the prohibitive costs and the extensive side effects of the treatment, an intense research for new direct-acting anti-HCV agents is taking place. Furthermore, NS3 protease inhibitors recently introduced into the market are not effective against all HCV subgenotypes. Thiosemicarbazones (TSCs) have shown antiviral activity against a wide range of DNA and RNA viruses. However, their extremely low aqueous solubility and high self-aggregation tendency often preclude their reliable biological evaluation *in vitro*. In this work, we investigated and compared for the first time the anti-HCV activity of two 1-indanone TSCs, namely 5,6-dimethoxy-1-indanone TSC and 5,6-dimethoxy-1-indanone N4-allyl TSC, and their inclusion complexes with hydroxypropyl-β-cyclodextrin (HPβ-CD) in Huh-7.5 cells containing the full-length and the subgenomic subgenotype 1b HCV replicon system. Studies of physical stability in culture medium showed that free TSCs precipitated rapidly and formed submicron aggregates. Conversely, TSC complexation with HPβ-CD led to more stable systems with minimal size growth and drug concentration loss. More importantly, both TSCs and their inclusion complexes displayed a potent suppression of the HCV replication in both cell lines with no cytotoxic effects. The mechanism likely involves the inhibition of non-structural proteins of the virus. In addition, findings suggested that the cyclodextrin released the drug to the culture medium over time. This platform could be exploited for the study of the drug toxicity and pharmacokinetics animal models.

1. Introduction

The infection by the hepatitis C virus (HCV) is a major global public health burden with an estimated rate of 170 million infections. Most of the infected individuals fail to clear the virus during the acute phase (Okuse et al., 2005). Viral persistence leads to chronic liver disease, a deterioration of the liver function that gradually progresses from steatosis and severe liver fibrosis to cirrhosis and hepatocellular carcinoma (HCC) (Lakatos et al., 2011). Extrahepatic diseases such as mixed cryo-globulinemia, non-Hodgkin lymphoma, central nervous system abnormalities and membrano-proliferative glomerulonephritis have been also associated with chronic HCV infection (Masarone and Persico, 2011). Furthermore, it is now widely recognized that chronic hepatitis C constitutes a metabolic disease strongly associated with insulin resistance and diabetes mellitus type 2 (Hung et al., 2011). Remarkable progress has been achieved in the treatment of the infection since the immune-modulator agent interferon was first used more than 20 years ago (Shiffman, 2011). To date, the standard therapy comprises a combination of PEGylated-interferon alpha (*PEG IFN-alpha*) and the antiviral ribavirin (RBV) (Pawlowska, 2011). Treatment duration and efficacy are intimately associated with the HCV genotype (Poordad and Khungar, 2011). At least six major HCV genotypes (designated 1–6) and more than 80 subgenotypes (named

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a, b, c, etc.) have been described based on the genomic sequence heterogeneity (Ashfaq et al., 2011). Subgenotypes 1a and 1b are the most common, accounting for about 60% of the infections worldwide. The currently available therapeutic options are very limited, especially for genotype 1 virus. Sustained virologic response (SVR), defined as an undetectable HCV RNA levels 24 weeks after the cessation of antiviral therapy, is attained in 40–50% of genotype 1-infected patients treated with *PEG IFN-alpha* and RBV (1000 or 1200 mg per day) for 48 weeks. Conversely, genotype 2- or 3-infected patients demonstrate SVR between 70% and 80% after receiving the same combination of drugs at a lower dose (800 mg per day) for 24 weeks (Rosen, 2011). This pharmacotherapy is expensive and substantial deleterious side effects seriously compromise patient compliance, reducing the chance for SVR (Poordad and Khungar, 2011). Furthermore, not all the chronic HCV patients are candidates for this therapy, underscoring the urgent need of an extended and comprehensive research for new direct-acting anti-HCV agents (DAA), as a novel antiviral strategy (Hunyadi, 2011). Several DAAs that inhibit NS3/NS4A serine-proteases, RNA-dependent NS5B polymerase and NS5A methyltransferase have been identified and entered the final steps of clinical development (Soriano et al., 2011). Moreover, some of them have already reached the pharmaceutical market; e.g., NS3 serine protease inhibitors telaprevir (tradenames Incivek® and Incivo®, joined development of Vertex and Johnson & and Johnson) and boceprevir (Victrelis®, Merck & Co., Inc.) (Soriano et al., 2011).

The therapeutic potential of thiosemicarbazones (TSCs) was first reported in the mid 1940s with *in vitro* assays against *Mycobacterium tuberculosis* (Domagk et al., 1946) and owing to their versatile chemistry, research has progressed to the design and synthesis of a broad spectrum of derivatives with antineoplastic (Iakovidou et al., 2001), antibacterial (Sriram et al., 2007), antifungal (Halve et al., 2008), antiprotozoal (Du et al., 2002) and antiviral (Pelosi et al., 2010) activity. Moglioni and coworkers designed different 1-indanone TSCs that displayed potent *in vitro* activity against: (i) bovine viral diarrhea virus (BVDV) (Finkielsztein et al., 2008; Castro et al., 2011), (ii) *Trypanosoma cruzi* (the causative agent of Chagas disease) (Caputto et al., 2011), and (iii) leukemias (Gómez et al., 2011). Among these derivatives, 5,6-dimethoxy-1-indanone TSC (Fig. 1A) was more effective than RBV against BVDV (Finkielsztein et al., 2008; Castro et al., 2011).

The main disadvantage of TSCs is that owing to their poor intrinsic aqueous solubility (1.5–13.0 μg/mL) and their innate self-aggregation tendency, they precipitate rapidly in water and water:dimethyl sulfoxide (water:DMSO) (Glisoni et al., 2010); water:DMSO (98:2) is usually used as TSC solvent in biological assays *in vitro*. This behavior results in unreliable antiviral activity data (Finkielsztein et al., 2008). We recently conducted a very comprehensive study on the solubilization and physical stabilization of these compounds in aqueous medium by means of complexation with different natural and chemically-modified cyclodextrins (CDs) (Glisoni et al., 2012). The solubility of the TSCs was increased up to 215 times and complexation effectively maintained the drug in solution for at least one week.

In this work, we investigated the anti-HCV activity of 5,6-dimethoxy-1-indanone TSC and 5,6-dimethoxy-1-indanone N4-allyl TSC and their inclusion complexes with hydroxypropyl-β cyclodextrin (HPβ-CD) in full-length (FL) and subgenomic (SG) HCV genotype 1b replicon systems. The former was previously assessed in BVDV (Castro et al., 2011), while the latter was evaluated for the first time. A potent suppression of HCV replication in both replicon systems was observed, strongly suggesting that the inhibitory mechanism involves non-structural proteins.

2. Materials and methods

2.1. Materials

5,6-dimethoxy-1-indanone TSC and 5,6-dimethoxy-1-indanone N4-allyl TSC are named TSC1 and TSC2, respectively (Fig. 1A) and were synthesized and purified as depicted elsewhere (Glisoni et al., 2010). HPβ-CD (Cavasol® W_7 HP; molar substitution, MS, per anhydro glucose unit of 0.65; average molecular weight, M_W, of 1400 g/mol; Wacker-Chemie AG, München, Germany) was a gift of SAFER S.A.C.I.F (Buenos Aires, Argentina) (Fig. 1B). All the solvents were of analytical grade and used without further purification.

2.2. Cell culture

The antiviral activity was assessed in HCV replicon systems Con1/FL-Neo(I) and Con1/SG-Neo (I) (Huh-7.5 cells containing the full-length (FL) and the subgenomic (SG) subgenotype 1b HCV replicon, BB7, respectively) generously donated by Dr. Charles M. Rice (Rockefeller University, New York, NY, USA) (Blight et al., 2002). Cultures were maintained in a sub-confluent state in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies Corp.,

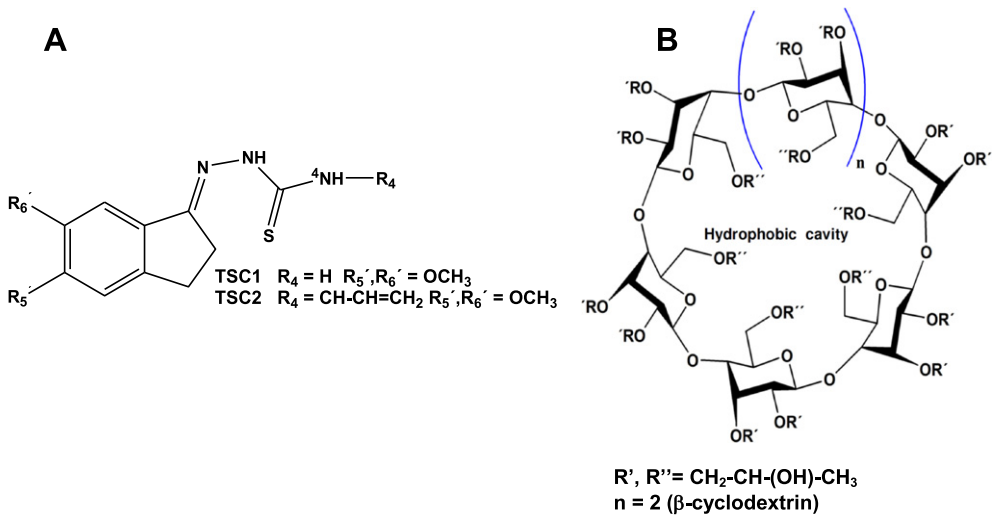

Fig. 1. Chemical structure of (A) TSC1 and TSC2 and (B) 2-hydroxypropyl-β-cyclodextrin included in the anti-HCV assays on both FL and SG replicon systems.

Carlsbad, CA, USA) containing 10% heat-inactivated fetal calf serum (FCS, Life Technologies Corp.) and geneticin (G418, 750 μg/mL, Life Technologies Corp.). All cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere. Cells were harvested by trypsinization and the number of live cells was determined with trypan blue (Life Technologies Corp.).

2.3. Preparation of TSC/HPβ-CD inclusion complexes

The preparation of TSC/HPβ-CD inclusion complexes was conducted by the co-solvent method, as previously reported (Glisoni et al., 2012). Briefly, HPβ-CD was dissolved in methanol at the desired concentration, the corresponding TSC was dissolved in chloroform:acetone (1:1, TSC1) or methanol:acetone (1:1, TSC2) and both solutions were thoroughly mixed over 15 min under magnetic stirring, at 25 °C. Organic solvents were removed under vacuum by means of a rotary evaporator (15 min, 70–90 °C, Fbr®, Decalab S.R.L, Buenos Aires, Argentina) to obtain white powders. Then, powders were solubilized in buffered saline solution (PBS, pH 7.4) at 2X the desired HPβ-CD final concentration and filtered (0.22 μm, Millipore Ireland B.V., Carrigtwohill, Co., Cork, Ireland) in a laminar flow. The final HPβ-CD concentrations in the different complexes were 0.25%, 0.5%, 1%, and 1.5% (w/v). To assess the antiviral activity of TSC/CD complexes against HCV, solutions were diluted (1:1 or 1:2 for TSC2/HPβ-CD, 13.0 μM/0.5% w/v final concentration) using DMEM containing 5% heat-inactivated FCS without G418. Thus, TSC/CD complexes yielded final HPβ-CD concentrations of 0.125%, 0.25%, and 0.5% (w/v), in the culture medium. TSC1/HPβ-CD and TSC2/HPβ-CD, 57.0 μM/0.5 and 3.0% w/v final concentration, respectively was prepared by solubilizing directly the complex in DMEM (5 mL) supplemented with 5% FCS.

2.4. Physical stability of TSC/HPβ-CD

As abovementioned, TSCs tend to self-aggregate and precipitate in aqueous media (Glisoni et al., 2010, 2012). To evaluate their behavior under the specific conditions of the biological assays and to ensure constant concentrations over time, solutions of free TSC in DMEM:PBS:DMSO (66:33:1) and TSC/HPβ-CD inclusion complexes in DMEM or PBS:DMEM (1:1 or 1:2) were prepared as described above. TSC concentrations in solution were monitored by UV–Visible spectrophotometry (λ_{max} of 329 and 330 nm for TSC1 and TSC2, respectively; CARY [1E] UV–Visible Spectrophotometer, Varian, Palo Alto, CA, USA) (Glisoni et al., 2010, 2012) over 24 h, at 37 °C. Concentrations were calculated by interpolating the absorbance of each sample in a calibration curve built in water:DMSO (98:2) covering the range between 7.5 and 75.0 μM, for both compounds (Glisoni et al., 2010, 2012). DMEM:PBS:DMSO (66:33:1) and DMEM or PBS:DMEM (1:1 or 1:2) were used as blank.

2.5. Cytotoxicity studies

Cytotoxicity was assessed prior to the antiviral activity. Both FL and SG replicon systems were exposed over 96 h to the following samples: (i) TSC1 and TSC2 (13.0–57.0 μM) in DMEM:PBS:DMSO (66:33:1); (ii) pristine HPβ-CD (0.125–3.0% w/v) in PBS:DMEM (1:1); (iii) TSC1/HPβ-CD (0.125–0.5% w/v final CD concentration) in DMEM or PBS:DMEM (1:1) and TSC2/HPβ-CD (0.125–3.0% w/v final CD concentration) in DMEM or PBS:DMEM (1:1 or 1:2). After 96 h, cells were harvested by trypsination and analyzed by flow cytometry using annexin V fluorescein isothiocyanate (AV-FITC) together with propidium iodide (PI) (FITC Annexin V Apoptosis Detection Kit I BD Pharmigen®, BD Biosciences, Franklin Lakes, NJ, USA). This staining distinguished between viable cells (AV-/PI-), early apoptotic cells (AV+/PI-) and late apoptotic/necrotic cells (AV-FITC+/PI+), as previously described (Cuestas et al., 2011). In all cases, flow cytometry was performed using a FACScan cytometer (BD Biosciences). A minimum of 10,000 events were acquired gating the forward and side scatters to exclude cell debris and analyzed in FL-1 and FL-2 whichever was applicable. Cytotoxicity was also assessed by the quantification of the activity of hepatic transaminases (aspartate aminotransferase [AST] and alanine aminotransferase [ALT]) in the supernatants of cell cultures by means of the IFCC kinetic method. AST/ALT ratios were calculated and compared to the untreated controls. Assays were conducted in triplicate for each concentration. All data are expressed as means ± S.D. of at least three independent experiments.

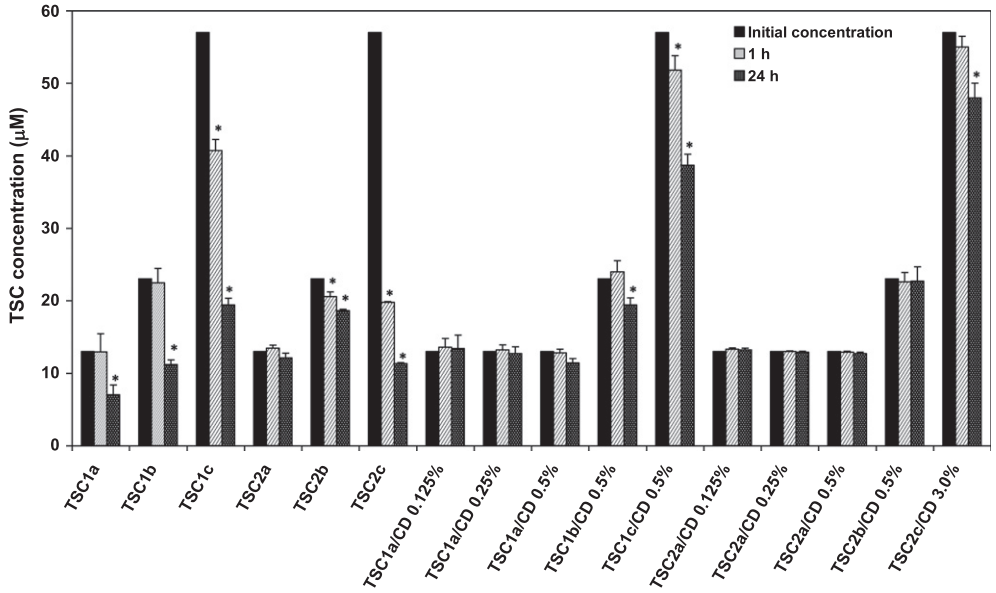

Fig. 2. Physical stability of free TSCs and TSC/HPβ-CD inclusion complexes in culture media, at 37 °C, as determined by UV spectrophotometry. Initial concentrations of TSCa, TSCb and TSCc were 13.0, 23.0 and 57.0 μM in DMEM:PBS:DMSO (66:33:1), respectively. The same initial TSC concentrations were used in the different TSC/CD complexes. The concentration of CD in DMEM or PBS:DMEM (1:1 or 1:2) is expressed in % w/v. All data are expressed as mean ± S.D. of at least three independent experiments. *Statistically significant decrease of TSC concentration when compared to the theoretical TSC concentrations at day 0 ($P < 0.05$).

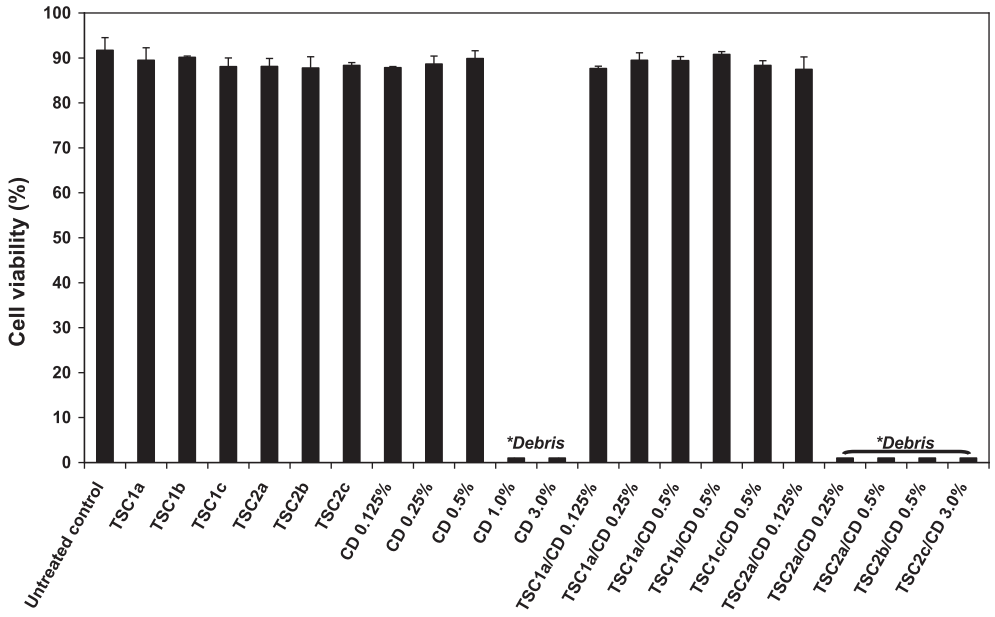

Fig. 3. Cytotoxicity of free TSC, pristine HPβ-CD and TSC/HPβ-CD inclusion complexes determined, by flow cytometry. Initial concentrations of TSCa, TSCb and TSCc were 13.0, 23.0 and 57.0 μM in DMEM:PBS:DMSO (66:33:1), respectively. The same initial TSC concentrations were used in the different TSC/CD complexes. The concentration of CD in DMEM or DMEM:PBS (1:1 or 1:2) is expressed in% w/v. Plots represent the average viability of FL and SG replicon systems. All data are expressed as mean ± S.D. of at least three independent experiments. *_Debris:_* Samples showed substantial cell debris and were not analyzed by flow cytometry. **Statistically significant decrease of viability when compared to untreated controls ($P < 0.05$).

2.6. Antiviral activity

Antiviral activity for each free TSC and TSC/CD complex was determined as previously described (Okuse et al., 2005). Briefly, replicon systems were maintained as sub-confluent cultures on 6-well plates. The following samples (2 mL) were added to cell cultures, 24 h after seeding: (i) TSC1 and TSC2 (13.0, 23.0 and 57.0 μM) in DMEM:PBS:DMSO (66:33:1), (ii) pristine HPβ-CD (0.125%, 0.25% and 0.5% w/v) in PBS:DMEM (1:1) and (iii) TSC1/HPβ-CD (13.0 μM/0.5%, 23.0 μM/0.5%, 57.0 μM/0.5%, 13.0 μM/0.25% and 13.0 μM/0.125% w/v) and TSC2/HPβ-CD (13.0 μM/0.125% w/v) in DMEM or PBS:DMEM (1:1). Samples were completely renewed every 24 h over 72 h (3 replacements), using fresh medium supplemented with 5% FCS without G418 in all cases (to eliminate potential cell loss due to the reduction of HCV replicon copy number and G418 resistance). Twenty-four hours after the last replacement of sample-containing medium, the antiviral activity was measured by quantifying HCV RNA levels in supernatants by RT-real time PCR using the COBAS® AmpliPrep/COBAS® TaqMan® HCV Quantitative Test (Roche, Branchburg, NJ, USA). Viral RNA levels were normalized to each untreated control (100% HCV RNA levels = 0% antiviral activity). Assays were conducted in triplicate for each TSC, CD and TSC/CD complex concentration. All data are expressed as means ± S.D. of at least three independent experiments.

Table 1
AST/ALT ratio in supernatants from Huh-7.5 cells containing the full-length (FL) and the subgenomic (SG) genotype 1b HCV replicon systems, either exposed to free TSCs, pristine HPβ-CD or TSC/HPβ-CD complexes over 96 h. All data are expressed as mean ± S.D. of at least three independent experiments. *Statistically significant decrease of AST/ALT with respect to untreated controls ($P < 0.05$).

Sample	AST/ALT (±S.D.)	
	FL	SG
Untreated control	8.3 (0.9)	6.0 (1.0)
TSC1 13.0 μM[a]	2.5* (0.5)	3.5* (0.5)
TSC1 23.0 μM[a]	3.0*(0.3)	3.5* (0.5)
TSC1 57.0 μM[a]	4.0* (0.4)	3.0* (0.4)
TSC2 13.0 μM[a]	1.0* (0.5)	3.0* (0.6)
TSC2 23.0 μM[a]	2.0* (0.5)	1.0* (0.4)
TSC2 57.0 μM[a]	4.0* (0.5)	2.0* (0.6)
HPβ-CD 0.125%[b]	4.5* (0.7)	4.0* (0.8)
HPβ-CD 0.25%[b]	2.5* (0.7)	2.0* (0.5)
HPβ-CD 0.5%[b]	2.5* (0.7)	3.0* (0.2)
TSC1/HPβ-CD 13.0 μM/0.125%[b]	5.0* (0.7)	2.0* (0.7)
TSC1/HPβ-CD 13.0 μM/0.25%[b]	4.0* (0.6)	2.5* (0.6)
TSC1/HPβ-CD 13.0 μM/0.5%[b]	3.7* (0.5)	2.0* (0.3)
TSC1/HPβ-CD 23.0 μM/0.5%[b]	2.5* (0.4)	1.5* (0.5)
TSC1/HPβ-CD 57.0 μM/0.5%[b]	5.0* (0.7)	2.6* (0.2)
TSC2/HPβ-CD 13.0 μM/0.125%[b]	3.5* (0.6)	2.3* (0.8)
TSC2/HPβ-CD 13.0 μM/0.25%[b]	ND	ND
TSC2/HPβ-CD 13.0 μM/0.5%[b]	ND	ND
TSC2/HPβ-CD 23.0 μM/0.5%[b]	ND	ND
TSC2/HPβ-CD 57.0 μM/3%[b]	ND	ND

ND: Not determined due to the cytotoxicity of the inclusion complex and substantial cell debris.
[a] TSCs in DMEM:PBS:DMSO (66:33:1).
[b] Concentration of CD expressed in % w/v in the different TSC-free HPβ-CD and TSC/HPβ-CD samples.

2.7. Statistical analysis

Comparison of treatments against controls was made with ANOVA using Bonferroni's post-test (Bonferroni's multiple comparison test). The significance level chosen for statistical analysis was $P < 0.05$. The software used was GraphPad Prism version5.00 for Windows (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

3.1. Physical stability of TSC/HPβ-CD complexes

To evaluate the self-aggregation of TSCs under the specific conditions of the biological assays, solutions of free TSC in DMEM:PBS:DMSO (66:33:1) and TSC/HPβ-CD in DMEM or PBS:DMEM (1:1 or 1:2) were prepared and monitored over 24 h, at 37 °C. Free TSC1 displayed the lowest physical stability in the

Table 2
Anti-HCV activity of free TSCs, pristine HPβ-CD and TSC inclusion complexes on Huh-7.5 line containing the full-length (FL) or the subgenomic (SG) 1b HCV replicon, respectively. Viral RNA concentrations in untreated controls were considered 100%. All data are expressed as mean ± S.D. of at least three independent experiments. *Statistically significant decrease of total HCV RNA level with respect to untreated controls ($P < 0.05$).

Sample	Total HCV RNA levels % (±S.D.)	
	FL	SG
Untreated control	100.00 (11.68)	100.00 (14.54)
TSC1 13.0 μM[a]	0.56* (0.14)	0.42* (0.06)
TSC1 23.0 μM[a]	0.34* (0.08)	0.25* (0.05)
TSC1 57.0 μM[a]	0.66* (0.17)	1.65* (0.41)
TSC2 13.0 μM[a]	0.23* (0.05)	0.24* (0.06)
TSC2 23.0 μM[a]	0.36* (0.07)	0.25* (0.05)
TSC2 57.0 μM[a]	ND	ND
HPβ-CD 0.125%[b]	63.08* (12.47)	79.40* (1.95)
HPβ-CD 0.25%[b]	60.75* (12.45)	83.07* (1.75)
HPβ-CD 0.5%[b]	56.68* (12.18)	72.86* (2.40)
TSC1/HPβ-CD 13.0 μM/0.125%[b]	18.25* (3.65)	16.59* (0.49)
TSC1/HPβ-CD 13.0 μM/0.25%[b]	17.27* (2.39)	15.88* (0.41)
TSC1/HPβ-CD 13.0 μM/0.5%[b]	15.94* (2.87)	15.61* (0.50)
TSC1/HPβ-CD 23.0 μM/0.5%[b]	3.50* (0.45)	1.87* (0.34)
TSC1/HPβ-CD 57.0 μM/0.5%[b]	5.48* (1.20)	6.98* (0.94)
TSC2/HPβ-CD 13.0 μM/0.125%[b]	24.56* (4.64)	23.53* (0.43)

ND: Not determined due to substantial precipitation of the corresponding TSC in culture medium at day 0.
[a] TSCs in DMEM:PBS:DMSO (66:33:1).
[b] Concentration of CD expressed in % w/v in the different HPβ-CD and TSC/HPβ-CD samples.

whole concentration range (13.0–57.0 μM in DMSO 1% v/v, see Fig. 2). TSC2 was unstable only at the greatest concentration (Fig. 2). Conversely, complexation of TSC1 and TSC2 resulted in greater physical stability, concentration loss being utmost 25.3% (Fig. 2). For example, the concentration of TSC1 (13.0 μM) decreased from 100.0% to 54.2% after 24 h, while TSC1/HPβ-CD (13.0 μM/0.125–0.25% w/v) remained soluble in the 96.2–98.5% range (Fig. 2). TSC2/HPβ-CD complexes also showed greater stability than free TSC2.

3.2. Cytotoxicity studies

Before investigating the antiviral activity of the different samples, we assessed the cytotoxicity of free TSCs, pristine HPβ-CD and both complexes on FL and SG genotype 1b HCV replicon systems. None of the free TSC solutions (13.0–57.0 μM) caused any significant decrease in the viability of FL and SG replicon systems, viability levels ranging between 86.4% and 90.1% (Fig. 3). These values were comparable to those of the controls that ranged between 88.9% and 94.5%. When HCV replicons were exposed to HPβ-CD, cytotoxicity strongly depended on the CD concentration and the incubation time. In fact, CD concentrations >0.5% w/v led to a high percentage of cell debris. At lower concentrations (0.125–0.5% w/v), cell viability was similar to the untreated controls (87.6–91.6%) (Fig. 3). As expected, the more prolonged the exposure, the greater the cytotoxicity of the CD. For example, HPβ-CD 1.0% w/v displayed a strong cytotoxicity after 48 h, while at a substantially greater concentration of 3.0% w/v cellular debris was observed already after 24 h (data not shown). In general, TSC1/HPβ-CD complexes that contained CD concentrations between 0.125% and 0.5% w/v did not show any significant viability loss in both replicon systems; viability values were between 87.1% and 91.4%. Interestingly, TSC2/HPβ-CD 13.0 μM/0.25–0.5% and 23.0 μM/0.5% w/v were cytotoxic to both HCV replicon systems as opposed to their separate components, suggesting a synergistic cytotoxicity (Fig. 3). TSC2 complexes containing 3% CD were also very cytotoxic.

To gain further insight into the cytotoxicity of the different samples, the activity of hepatocyte transaminases was quantified in the supernatants of cell cultures exposed over 96 h. Interestingly, AST/ALT ratio values determined in the supernatants of both replicon cells treated with TSCs or TSC/CD inclusion complexes were lower than those obtained in the untreated controls (Table 1). For example, untreated controls of FL and SG showed an AST/ALT ratio of 8.3 and 6.0, respectively, while values for TSC2 23.0 μM were 2.0 (FL) and 1.0 (SG). Complexes showed intermediate values between 1.5 and 5.0.

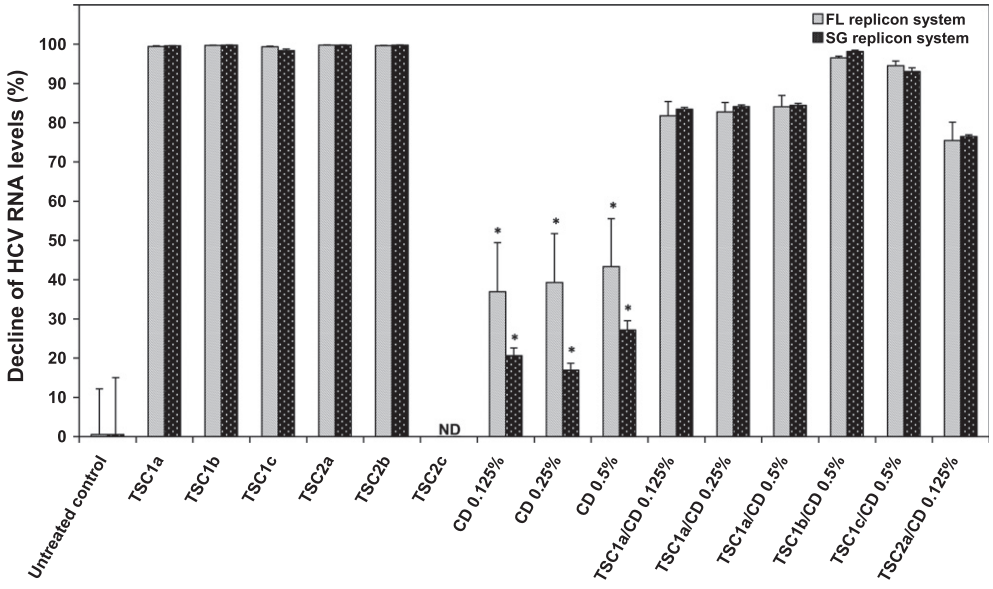

Fig. 4. Anti-HCV activity of free TSC, prisitine HPβ-CD and TSC/HPβ-CD inclusion complexes on FL and SG replicon systems after 96 h incubation. Initial concentrations of TSCa, TSCb and TSCc were 13.0, 23.0 and 57.0 μM in DMEM:PBS:DMSO (66:33:1), respectively. The same initial TSC concentrations were used in the different TSC/CD complexes. The concentration of CD in DMEM or PBS:DMEM (1:1) is expressed in % w/v. All data are expressed as means of the decline of HCV RNA levels (%) with respect to untreated control (100% viral load) ± S.D. of at least three independent experiments. ND: Not determined due to substantial precipitation of TSC in the culture medium at day 0. *Statistically significant difference between FL and SG replicon systems ($P < 0.05$).

3.3. Antiviral activity

The viral load in each individual assay after the exposure of the replicon systems to non-cytotoxic concentrations of free TSCs and TSC/HPβ-CD complexes were measured in all the supernatants and normalized to the levels measured in the untreated controls (Table 2). The SG replicon system employed in this study encodes for non-structural viral proteins, while FL codes for both structural and non-structural ones. These data indicated that the inhibition of non-structural proteins is one of the mechanisms promoting the antiviral activity of these TSCs. The sharp decline of the basal levels of HCV RNA upon exposure to free TSCs and TSC1 and TSC2/HPβ-CD inclusion complexes was equivalent in both FL and SG replicon systems (Fig. 4). For example, free TSC1 and TSC2 (13.0 and 23.0 μM) reduced the viral load in more than 99.4%. Free TSC1 57.0 μM was slightly less effective, though values remained above 98.3%. HPβCD was active to some extent, with declines of approximately 36.9–43.3% and 16.9–27.1% in FL and SG replicon systems, respectively. It is worth remarking that TSCs in complex with HPβ-CD were less active than the corresponding free TSCs with declines between 75.4% and 98.1% (Table 2, Fig. 4). However, the former remained soluble in aqueous medium, while the latter precipitated and the concentration was unknown (see above). Also here, differences between FL and SG were not statistically significant. Interestingly, the most active inclusion complex in both HCV replicon systems was TSC1/HPβ-CD (23.0 μM/0.5% w/v) (Table 2, Fig. 4).

4. Discussion

HCV is the etiologic agent of one of the most widespread infections and a key player in the development of liver cancer. The current pharmacotherapy is effective in a limited number of patients and its great cost compromises patient affordability. In this context, there is an urgent need for the discovery of more effective antivirals. A better understanding of viral dynamics and lifecycle has led to the discovery of several potential targets for the design of novel antiviral drugs. These drugs, collectively called DAA, include a range of inhibitors of NS3/NS4A serin-proteases, methyltransferase NS5A and RNA-dependent RNA polymerase NS5B, a crucial component of the replication complex (Halfon and Locarnini, 2011). NS3/NS4A serine-protease inhibitors are the most advanced in clinical development. Phase III clinical data of naïve and treatment-experienced patients infected with the HCV genotype 1 and treated with telaprevir and boceprevir, each one given in combination with PEG INF-alpha plus RBV, demonstrated SVR rates ranging between 66% and 75% and 59–66%, respectively (Vermehren and Sarrazin, 2011). These two antivirals have been already approved by the US-FDA for the treatment of HCV (Soriano et al., 2011).

Poor aqueous solubility represents one of the most remarkable biopharmaceutic hurdles in new drug development. It challenges not only the subsequent preclinical and clinical stages but also early *in vitro* biological assays, contributing to increase the drug attrition rates. CDs are oligosaccharides that combine a hydrophobic nano-cavity with a hydrophilic surface and they have been shown to improve the aqueous solubility and the physical stability of TSCs by reducing their self-aggregation tendency (Glisoni et al., 2012).

In this work, we described the anti-HCV activity of two TSCs derived from 5,6-dimethoxy-1-indanone and their TSC/HPβ-CD inclusion complexes in FL and SG HCV genotype 1b replicon systems. The employment of these cell lines allowed us to start to discern whether the antiviral activity relied on the inhibition of structural and/or non-structural proteins. Noteworthy, TSC1 was previously characterized as a compound that inhibits the viral RNA polymerase of BVDV (Castro et al., 2011). The uniqueness of this study is that we employed an *in vitro* model of the HCV *in vivo* infection and not a surrogate one such as BVDV.

The physical stability of free TSCs and TSC/HPβ-CD complexes was evaluated to select those samples that complied with a fundamental requirement of the biological assay: physical stability (and constant concentration) over 24 h; samples that formed crystals visible to the naked eye (e.g., free TSC2 57.0 μM) after 1 h were ruled out and not further investigated. Free TSC1 57.0 μM also tended to form aggregates though they were nanoscopic and thus this sample was assayed for cytotoxicity and antiviral activity. The lower physical stability of free TSC1 with respect to TSC2 in culture medium (Fig. 2) was in agreement with our previous studies, showing the greater self-aggregation tendency of this derivative in water by means of hydrophobic interactions involving the aromatic ring of 1-indanone residues (Glisoni et al., 2010). Even though, TSC2 was more stable at concentrations between 13.0 and 23.0 μM, a more concentrated solution rapidly precipitated and resulted in a final concentration that was 34.7% of the initial concentration (Fig. 2). This behavior correlated well with its greater experimental logP of 2.83 (Glisoni et al., 2010). Usually, TSC/CD complexes containing growing TSC content for constant CD concentrations became less physically stable (Fig. 2). Thus, the TSC solubility decrease was utmost 25.0% for TSC/CD complexes, while it was 50% or more for free TSCs. For example, TSC2 at the greatest concentration under study (57.0 μM) precipitated even after 1 h, while it remained in solution when it was complexed with HPβ-CD (TSC2/HPβ-CD 57.0 μM/3.0% w/v). This result was in agreement with our previous study (Glisoni et al., 2012). However, this HPβ-CD concentration was strongly cytotoxic and could not be assessed in assays of antiviral activity.

The cytotoxicity of CDs is a well investigated phenomenon and it is attributed to several factors including their scour effect on biological membranes, causing (i) death of different cell types during *in vitro* assays and (ii) systemic toxicity such as hemolysis, even at low concentrations (Matilainen et al., 2008). The hydroxypropylation of CD leads to a reduction of the cytoxicity because it alters the interaction of the CD with the constituents of the cell membrane (e.g., cholesterol and phospholipids) (Matilainen et al., 2008). HPβ-CD is generally regarded as a safe pharmaceutical excipient for the delivery of hydrophobic drugs (Gould and Scott, 2005). The exhaustive study of TSC complexation behavior using CDs was essential before the comprehensive evaluation of their cytotoxicity and antiviral activity in HCV cell constructs was carried out (Glisoni et al., 2012).

None of the free TSC solutions (13.0–57.0 μM) caused a significant decrease in viability of FL and SG replicon systems (Fig. 3). The intrinsic cytotoxicity of HPβ-CD was strongly dependent on the CD concentration and the incubation time. Our findings were in good agreement with previous reports showing that different cell types exposed to CDs for several hours tolerated well HPβ-CD concentrations of up to 0.5–1.0% w/v (Matilainen et al., 2008). Besides the CD concentration used, the incubation time played a central role and governed the cytotoxicity of the CD on FL and SG replicon systems (Matilainen et al., 2008; Hipler et al., 2007). The fact that TSC2/HPβ-CD complexes (13.0 μM/0.25–0.5% and 23.0 μM/0.5% w/v) were cytotoxic to both replicon systems as opposed to the free drug and the pristine CD, stressed the relevance of investigating this effect prior to any evaluation of the antiviral activity (Fig. 3). In addition, findings confirmed that these specific complexes should be considered new entities that are more prone to interact with cell membranes and damage them. Hepatic transaminases are usually used as a complementary indication of hepatic cell cytotoxicity (Cuestas et al., 2011). Interestingly, AST/ALT ratios in supernatants of both replicon systems exposed to free TSC and TSC/CD samples decreased with respect to untreated controls, suggesting

a normalizing effect of the liver cell function when the viral load was reduced.

The evaluation of the antiviral activity of RBV (Grancher et al., 2004), acyclovir (Bencini et al., 2008), ganciclovir (Nicolazzi et al., 2002) and cosalane (Udata et al., 2003) was possible after the preparation of complexes with CDs. Complexation of RBV improved the efficacy of the drug *in vitro* against measles virus (Grancher et al., 2004). Free TSCs displayed a remarkable anti-HCV activity expressed by the sharp decrease of HCV RNA levels by more than 99%, after 96 h. Complexes showed more moderate declines than the free TSC (Table 2, Fig. 4). This phenomenon would probably stem from the fact that, in the case of complexes, the effective free TSC concentration available in the cell culture medium and ready to be absorbed by the cells was smaller than in free TSC samples. In other words, CD served as an effective TSC reservoir that maintained TSC in solution and that gradually released it over time. This release profile is governed by the complex stability constant ($k_{1:1}$) that is a measure of the drug/CD interaction strength. In general, $k_{1:1}$ values between 50 and 2000 M^{-1} are appropriate for drug complexation and release (Brewster and Loftsson, 2007; Messner et al., 2009). Values <50 M^{-1} indicate weak drug/CD interactions and small complexation capacity, while values above 2000 M^{-1} are indicative of strong interactions that limit the release of the drug from the cavity (Mukne and Nagarsenker, 2004). TSC1/HPβ-CD and TSC2/HPβ-CD showed $k_{1:1}$ values of 123.8 and 186.2 M^{-1} (Glisoni et al., 2012). These values were in the acceptable range to effectively solubilize and stabilize TSC. However, they would lead to smaller concentrations in culture medium and to a less impressive decline in the viral RNA concentrations. Based on this, the slower release of TSC2 from a stronger complex and the smaller antiviral activity with respect to TSC1 was anticipated. On the other hand, TSC1/HPβ-CD (23.0 μM/0.5% w/v) displayed a strong anti-HCV activity in both replicon systems and it was almost as effective as the free drug. Pristine HPβ-CD also decreased the HCV RNA levels, though to a much lesser extent. However, a synergistic antiviral effect of TSC and CD in the complex was not apparent. These findings confirm that drug/CD complexes need to be considered as new entities and that a comprehensive analysis should be undertaken. The decrease of AST/ALT was concomitant to the decline of HCV-RNA levels in all the samples, strongly suggesting that the regardless of the extent, the inhibition of the viral replication normalizes the transaminase levels. This parameter could emerge as a useful indicator of antiviral activity in preliminary stages of screening. However, to assess the antiviral activity of new drugs, the measurement of extracellular (and in more advanced stages, intracellular) HCV RNA concentrations will be unavoidable.

5. Conclusions

The antiviral efficacy of two 1-indanone TSC derivatives and their inclusion complexes with HPβ-CD was demontrated using the human hepatoma cell line Huh-7.5 harboring either the FL or the SG HCV 1b subgenotype replicon systems. TSC/HPβ-CD inclusion complexes were more physically stable than free TSCs that precipitated rapidly in the culture medium. Free TSCs were slightly more effective than TSC1/HPβ-CD (23.0 μM/0.5% w/v), the most effective complex; the drug/CD constant playing a fundamental role. These findings suggested that complexes released the drug from the cavity over time and that the effective TSC concentration in the culture medium of TSC/CD complexes was slightly smaller than that in free TSC samples. On the other hand, complexation ensured the physical stability of the systems over the whole study and prevented drug precipitation. Finally, the low aqueous solubility also represents a drawback for the assessment of the toxicity and the pharmacokinetics in an animal model. Owing to the biocompatibility of HPβ-CD and its spread implementation as pharmaceutical excipient for administration by different routes, these complexes may constitute a useful nanotechnology platform to continue with the evaluation of the biopharmaceutic performance of these drugs *in vivo*.

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