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# Inhibitory effects of Pycnogenol® on hepatitis C virus replication

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ABSTRACT

Chronic hepatitis C virus (HCV) infection increases the risk of liver cirrhosis and hepatocellular carcinoma. In the last decade, the current standard HCV treatment, pegylated interferon and ribavirin, have limited efficacy and significant side effects. Novel direct acting antivirals show promise, but escape mutants are expected, along with potential side effects. Pycnogenol®, a French maritime pine extract, has been reported to have antioxidant and antiviral effects. Here, we evaluated the effect of Pycnogenol® on HCV replication.

Wild-type and protease inhibitor (VX-950; telaprevir)-resistant HCV replicon cells were treated with Pycnogenol®, Pycnogenol® and interferon-alpha, and ribavirin and telaprevir. Pycnogenol® effects on replication were also evaluated in HCV-infected chimeric mice.

Pycnogenol® treatment showed antiviral effects without cytotoxicity at doses up to 50 μg/mL. Pycnogenol® in combination with interferon-alpha or ribavirin showed synergistic effects. Moreover, Pycnogenol® inhibited HCV replication in telaprevir-resistant replicon cells; telaprevir and Pycnogenol® acted additively to reduce HCV RNA levels in wild-type HCV replicon cells without significantly increasing cytotoxicity. Pycnogenol® antiviral activity was higher than its components procyanidin and taxifolin. Further, treatment of infected chimeric mice with Pycnogenol® suppressed HCV replication and showed a synergistic effect with interferon-alpha. In addition, Pycnogenol® treatment resulted in dose-dependent reduction of reactive oxygen species in HCV replicon cell lines.

Pycnogenol® is a natural product that may be used to improve the efficacy of the current standard antiviral agents and even to eliminate resistant HCV mutants.



## 1. Introduction

Approximately 130–170 million people are chronically infected with HCV, leading to 54,000 deaths and 955,000 disability-adjusted life-years associated with acute HCV infection (Mohd Hanafiah et al., 2013). Chronic hepatitis C can lead to a large spectrum of diseases, including steatosis, fibrosis, cirrhosis, and hepatocellular carcinoma (Perz and Alter, 2006). To date, no protective vaccine is available for HCV infection; over the last decade, therapy has consisted of a 24–48-week course of peginterferon-alpha-2a (PEG-IFN-alpha-2a) or peginterferon-alpha-2b (PEG-IFN-alpha-2b) in combination with the guanosine analogue, ribavirin (RBV). The therapy leads to sustained virologic response (SVR) in 42–52%, 65–85%, and 76–82% of individuals infected with HCV genotype 1; 4, 5, or 6; and 2 or 3, respectively (Antaki et al., 2010; Hoofnagle and Seeff, 2006). The recently approved non-structural

*Abbreviations:* $CC_{50}$, 50% cytotoxic concentration; CI, combination index; DAA, direct acting antivirals; $EC_{50}$, 50% effective concentration; HCV, hepatitis C virus; $IC_{50}$, 50% inhibitory concentration; IRES, internal ribosome entry site; NS, non-structural protein; NF-kappa B, nuclear factor-kappa B; PC, procyanidin; PEG-IFN-alpha-2a, pegylated interferon-alpha-2a; PEG-IFN-alpha-2b, pegylated interferon-alpha-2b; RBV, ribavirin; PYC, Pycnogenol®; qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction; ROS, reactive oxygen species; SI, selectivity index.

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protein (NS) 3/4A protease inhibitors (PIs) boceprevir (approved by the FDA on May 13, 2011) and telaprevir (approved by the FDA on May 23, 2011), used in combination with PEG-IFN-alpha and RBV for HCV genotype 1 infections, have increased cure rates to approximately 70% (Bacon et al., 2011; Jacobson et al., 2011; Poordad et al., 2011). However, these triple-therapy regimens may result in unfavourable side effects and emergence of drug-resistant HCV (Bacon et al., 2011; Jacobson et al., 2011; Poordad et al., 2011), which may reduce virus susceptibility and applicability of current HCV triple therapies (Ozeki et al., 2011). Recently, two more effective compounds have been approved for HCV treatment: the protease inhibitor simeprevir (approved by the FDA in November, 2013) and the nucleotide polymerase inhibitor sofosbuvir (approved by the FDA on December 6, 2013). Among patients infected with HCV, less than 10% are treated and cured, and the major challenge in controlling HCV infections is the identification of those already infected, most of whom are situated in the poorest regions of the world (Thomas, 2013), and to find the most effective, tolerable and affordable direct acting antivirals (DAA) combination that can cure people in the shortest period (Poveda et al., 2014). In the NEUTRINO phase III trial of treatment-naive patients, 12 weeks of triple combination therapy with sofosbuvir (400 mg) once daily resulted in SVR rates of 89% in patients with HCV genotype 1 (92% for subtype 1a and 82% for subtype 1b), and 96% in patients with genotype 4 (Lawitz et al., 2013). Moreover, in the FISSION trial of HCV-2/3 treatment-naive patients receiving sofosbuvir/RBV for 12 weeks, 95% of patients with genotype 2 and 56% of patients with genotype 3 achieved an SVR (Lawitz et al., 2013). In addition, most DAA agents are characterised by a low genetic barrier to the development of resistance, except sofosbuvir, which showed a very high resistance barrier. This is the reason most current DAA-based therapies under evaluation must be co-administered with either PEG-IFN-alpha and ribavirin or different compounds belonging to different DAA classes (Poveda et al., 2014).

Pycnogenol® (PYC; trademark of Horphag Research, Geneva, Switzerland) is a French maritime pine extract produced from the outer bark of *Pinus pinaster* ssp. *atlantica*, and is generally considered safe for human use (American Botanical Council, 2010). The main PYC constituents are procyanidins (68.4%), taxifolin (21.87%), ferulic acid (3.70%), catechin (2.53%), and caffeic acid (3.51%) (Lee et al., 2010). PYC has been reported to have antioxidative and anti-inflammatory effects, and to reduce cardiovascular risk factors associated with type 2 diabetes (Maimoona et al., 2011; Zibadi et al., 2008). A recent report suggests that PYC can inhibit encephalomyocarditis virus replication in the mouse heart by suppressing expression of proinflammatory cytokines, and genes related to cardiac remodelling and mast cells (Matsumori et al., 2007). PYC has also been reported to inhibit binding of human immunodeficiency virus type-1 to host cells, and to cause other significant changes, including increased expression of manganese superoxide dismutase (Feng et al., 2008).

HCV gene expression elevates reactive oxygen species (ROS) levels via calcium signalling. In addition, HCV Core, NS3, and NS5A proteins have all been shown to induce oxidative stress (Choi et al., 2004). The reported link between HCV and oxidative stress makes this pathway a promising anti-HCV therapeutic strategy. To date, however, the effect of PYC on HCV infection has not been investigated. This study evaluated the inhibitory effects of Pycnogenol® on HCV replication *in vitro* and *in vivo*.

## 2. Materials and methods

### 2.1. Cell culture and reagents

Genotype 1b HCV subgenomic replicon cell lines, R6FLR-N (R6, genotype 1b, strain N) (Watanabe et al., 2006), FLR3-1 (genotype 1b, Con-1) (Sakamoto et al., 2005) and Rep JFH Luc3-13 genotype 2a (Takano et al., 2011), strain JFH-1 (Wakita et al., 2005) (Supplementary Fig. 1) were cultured at 37 °C (5% $CO_2$) in Dulbecco's modified Eagle medium-GlutaMAX-I (DMEM-GlutaMAX-I; Invitrogen, Carlsbad, CA, USA) containing 10% foetal bovine serum and 0.5 mg/mL G418 (Invitrogen, Carlsbad, CA, USA) (Sakamoto et al., 2005; Watanabe et al., 2006). The JFH-1/K4 cell line, which comprises HuH-7 cells persistently infected with the HCV JFH-1 strain, was maintained in DMEM with 10% FCS (Takano et al., 2011).

PYC was supplied by Horphag Research Co., Pegylated IFN-alpha-2a was obtained from Chugai Pharmaceutical Co., Japan.

### 2.2. HCV replicon cell reporter assay

Cells were seeded into 96-well plates ($5 \times 10^3$/well). After incubation for 24 h at 37 °C (5% $CO_2$), the medium was removed and replaced with growth medium containing serial dilutions of PYC, IFN-alpha, RBV, telaprevir or simeprevir (Janssen Pharma Co., Tokyo, Japan). After 72 h, luciferase activity was measured using the Bright-Glo luciferase assay kit (Promega, Madison, WI). Measurements were made in triplicate using an AccuFLEX Lumi 400 luminometer (Aloka, Tokyo, Japan), and the results expressed as the average percentage of the control.

### 2.3. Generation of telaprevir-resistant replicon cell lines and analysis

Telaprevir-resistant R6FLR-N subgenomic replicon cell lines were established as described previously (Katsume et al., 2013). Briefly, wild-type R6FLR-N replicon cells were seeded in 10-cm dishes in the presence of 0.5 mg/mL G418 and treated with telaprevir. The cells were incubated for 51 days with no-compound control or telaprevir (1.8 μM and 2.7 μM serially diluted in media). Fresh media and telaprevir were added every 3 days. Most cells incubated with 2.7 μM telaprevir died; however, after 3 weeks small colonies started to appear and were expanded for 4 weeks. Deep sequencing was performed as described previously (Katsume et al., 2013) and revealed a mutation profile in NS3 (V36A, T54V and A156T) and NS5A (Q181H, P223S and S417P) which confer resistance to telaprevir. Resistant replicon cells were seeded at $5 \times 10^3$/well. After incubation for 24 h at 37 °C (5% $CO_2$), culture medium was removed and replaced with growth medium containing serial dilutions of PYC or telaprevir alone or in combination. After 72 h, luciferase activity was determined using the Bright-Glo luciferase assay kit (Promega, Madison, WI, USA). Measurements were made in duplicate using a GloMax-Multi detection system (Promega, Madison, WI, USA). Cytotoxicity was measured using WST-8 cell counting kit (Dojindo, Kumamoto, Japan). Western blot analysis was performed, as described previously (Nishimura et al., 2009). Briefly, HCV replicon cells ($2 \times 10^5$) were grown in a 60-mm cell culture dish. After 24 h, cells were treated with PYC for 72 h. Cells were collected and lysed with radioimmunoprecipitation buffer (1% sodium dodecyl sulphate, 0.5% Nonidet P-40, 150 mmol NaCl, 0.5 mmol ethylenediaminetetraacetic acid, 1 mmol dithiothreitol, and 10 mmol Tris, pH 7.4). Total protein (30 μg) was electrophoresed on a 12% sodium dodecyl sulphate–polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Billerica, MA, USA). HCV NS3 and NS5B proteins were detected using rabbit NS3 (R212) polyclonal antibody or anti-NS5B (5B14) monoclonal antibody. Beta-actin was detected using an actin monoclonal antibody (Sigma, St. Louis, MO, USA).

### 2.4. Quantitative real-time polymerase chain reaction

Quantification of HCV RNA was performed using real-time reverse transcription polymerase chain reaction (qRT-PCR) based