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SELECTIVE INHIBITION OF HEPATITIS C VIRUS REPLICATION BY ALPHA-ZAM, A *NIGELLA SATIVA* SEED FORMULATION

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Abstract

Background: Hepatitis C virus (HCV) infection became curable because of the development of direct acting antivirals (DAAs). However, the high cost of DAAs has greatly impeded their potential impact on the treatment of HCV infection. As a result, hepatitis C will continue to cause substantial morbidity, and mortality among chronically infected individuals in low and middle income countries. Thus, urgent need exists for developing cheaper drugs available to hepatitis C patients in these countries.
Materials and Methods: Alpha-zam, an indigenous herbal formulation from *Nigella sativa* seed, was examined for its anti-HCV activity and cytotoxicity in genotype 1b HCV replicon cells. The antiviral activity was determined by luciferase expression and viral RNA synthesis, while the cytotoxicity was assessed by viable cell number and glyceraldehyde-3-phosphate dehydrogenase RNA synthesis in the replicon cells.
Results: Alpha-zam was found to be a selective inhibitor of HCV replication. The 50% effective dilution and 50% cytotoxic dilution of Alpha-zam were 761- and < 100-fold, respectively, in the subgenomic replicon cells LucNeo#2. Its selective inhibition of HCV was also confirmed by HCV RNA levels in LucNeo#2 and in the full-genome HCV replicon cells NNC#2 using real-time reverse transcriptase polymerase chain reaction. Furthermore, the anti-HCV activity of Alpha-zam was not due to the induction of interferon.
Conclusion: Alpha-zam selectively inhibits HCV replication and therefore has potential for a novel antiviral agent against HCV infection.

Keywords: Alpha-zam, chronic hepatitis, hepatitis C virus, antiviral assay, *Nigella sativa*

Introduction

Acute hepatitis C virus (HCV) infection is rarely associated with life-threatening disease, with 15–45% of infected persons recovering within 6 months without any treatment (WHO, 2015). However chronic infection develops in the remaining 55-85% out of which 15-30% eventually progress to liver cirrhosis after many years of persistent virus carriage. Though Hepatitis C virus is found worldwide, the prevalence is higher (≥ 3.5%) in developing countries of Africa, Middle East, Central and Eastern Asia, where majority of the chronically infected individuals are found. Chronic carriage of HCV currently affects 3% of the global population with 3-4 million new infections and 350,000 deaths occurring every year; however in 2013 alone, 343,000 and 358,000 deaths were due to HCV associated liver cancer and cirrhosis respectively (Madava et al., 2002; WHO, 2004; Anonymous 2016a). Although the reason for the varying rate is unknown, men are 2-fold more likely to die from untreated chronic liver disease and cirrhosis than women (Rogers et al., 2010). The risk for cirrhosis is exacerbated by exposure to alcohol and concurrent infection with human immunodeficiency virus (HIV), hepatitis B virus (HBV), and schistosoma. While HCV is a hepatotropic virus, extrahepatic conditions have been documented in patients with chronic infections. These included heamatologic, autoimmune, renal, dermatologic, endocrinal, neuromuscular, and neuropsychiatric conditions (Khattab et al., 2010). Also explanation for the conditions has been supported by a growing evidence of HCV replication in extrahepatic tissues including peripheral blood mononuclear cells (Goutagny et al., 2003; Radkowski et al., 2005).

Persistent HCV infection has been a major risk factor for hepatocellular carcinoma (HCC) development (about 2-6% per year) in patients with cirrhosis, mainly through indirect pathways, which include chronic inflammation, cell death, cell proliferation, and induction of free radicals (Sangiovanni et al., 2004; Farinati et al., 2007). Currently, an estimated 25% of HCC are due to HCV infection worldwide with higher burden of disease in highly endemic countries like Japan (Perz et al., 2006) while a prospective study documented a significantly higher risk of HCC among cirrhotic patients infected with the genotype 1b strain (Bruno et al., 2007). Although the incidence rate of HCV infection is decreasing in the developed world, deaths from liver disease will continue to increase over the next 20 years as those who were infected by transfusion and organ transplantation before HCV testing become apparent (Razavi et al., 2013).

Treatment of hepatitis C for virus eradication and non-progression to decompensated liver diseases is achievable and highly recommended for all with chronic infection. The recovery rate however is determined by the strain of the infecting virus, the type of treatment and its early institution (WHO, 2015). An earlier treatment for hepatitis C which combined interferon and ribavirin effectively resolved the infection leading to a cure in 50% of the treated individuals; though frequently associated with life threatening adverse reactions. (WHO, 2015). The search for other less toxic treatments led to the development of new compounds called the direct antiviral

agents (DAAs), which are better tolerated with fewer or no side effects and producing a cure rate of 90% (WHO, 2015). However, the economic costs of treating hepatitis C are significantly huge and enormous to both the individual and the society, resulting in its non-affordability and non-utilization by patients.

The course of an antiviral treatment for hepatitis C for 12 weeks by the Gileads sofosbuvir currently costs $84,000-$94,000, £35,000, and €41,000 in the USA, United Kingdom, and Germany, respectively (Anonymous, 2016b). Hence, the desirable therapeutic benefit of the highly effective DAAs is currently beyond most health systems, and consequently, patients are unable to access treatment even in the high-income countries. This is more pronounced in the poor resource countries, where the disease burden alongside other poverty associated factors are responsible for causing substantial morbidity and mortality. Hence, the urgent need to screen for and develop effective and cheaper alternatives for access to treatment globally, if deaths from preventable cirrhosis and liver cancer would be minimized in the future.

Materials and Methods

Compounds

The preparation of Alpha-zam has been described elsewhere (Onifade et al., 2010). It was dissolved in water and stored at -20°C until use. The HCV protease inhibitor telaprevir (TLV) was purchased from Funakoshi, Tokyo, Japan and dissolved in dimethyl sulfoxide (DMSO) at 20 mM or higher.

Cells

The subgenomic HCV replicon cells carrying luciferase gene as a reporter LucNeo#2 and the full-genome NNC#2 (Goto et al., 2006; Ishii et al., 2006) were grown and cultured in Dullbecco's modified Eagle medium with high glucose (Nakalai Tesque, Kyoto, Japan) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin G, 100 µg/ml streptomycin and 1 mg/ml G418 (Nakalai Tesque).

Cytotoxicity assays

The assay methods for drug cytotoxicity have been described previously (Salim et al, 2011). Briefly, LucNeo#2 and NNC#2 cells (5×10^3 cells/well) were cultured in a 96-well plate in the absence of G418 and in the presence of various dilutions of Alpha-zam. After incubation for 3 days at 37ºC, the number of viable cells was determined by a dye method using the water soluble tetrazolium Cell Counting Kit-8® (Dojindo Laboratories, Kumamoto, Japan), according to the manufacturer's instructions. The cytotoxicity of Alpha-zam was also evaluated by the inhibition of host cellular mRNA synthesis. The cells were washed with PBS, treated with lysis buffer in TaqMan® Gene Expression Cell-to-CT™ kit (Applied Biosystems, Carlsbad, CA), and the lysate was subjected to real-time RT-PCR using a TaqMan® RNA control reagent (Applied Biosystems).

Antiviral assays

The anti-HCV activity of alpha-zam was determined by a modification of the described methods (Windisch et al, 2005; Salim et al, 2011). Briefly, LucNeo#2 cells (5×10^3 cells/well) were cultured in a 96-well plate in the absence of G418 and in the presence of various dilutions of Alpha-zam. After incubation for 3 days at 37ºC, the culture medium was removed, and the cells were washed twice with PBS. Lysis buffer was added to each well, and the lysate was transferred to the corresponding well of a nontransparent 96-well plate. The luciferase activity was measured by addition of the luciferase reagent in a luciferase assay system kit (Promega, Madison WI) using a luminometer with automatic injectors (Berthold Technologies, Bad Wildbad, Germany).

The activity of Alpha-zam was also determined by the inhibition of HCV RNA synthesis in LucNeo#2 and NNC#2 cells. The cells (5×10^3 cells/well) were cultured in a 96-well plate in the absence of G418 and in the presence of various dilutions of Alpha-zam. After incubation for 3 days at 37ºC, the cells were washed with PBS, as described above, treated with lysis buffer in TaqMan® Gene Expression Cell-to-CT™ kit (Applied Biosystems), and the lysate was subjected to real-time RT-PCR, according to the manufacturer's instructions. The 5'-untranslated region of HCV RNA was quantified using the sense primer 5'-CGGGAGAGCCATAGTGG-3', the antisense primer 5'-AGTACCACAAGGCCTTTCG-3', and the fluorescence probe 5'-CTGCGGAACCGGTGAGTACAC-3' (Applied Biosystems).

Expression of 2', 5'-oligoadenylate synthetase 2

The ability of Alpha-zam to induce the expression of 2', 5'-oligoadenylate synthetase (OAS)-2 was determined in LucNeo#2 and NNC#2 cells. The cells (5×10^3 cells/well) were cultured in a 96-well plate in the absence of G418 and in the presence of the various concentration of Alpha-zam. The cells were treated as described above and subjected to real-time RT-PCR for detection of a part of OAS2 RNA using OAS2 TaqMan® Gene Expression Assay (Applied Biosystems).

Results

When Alpha-zam was examined for its inhibitory effect on HCV replication in LucNeo#2 cells, Alpha-zam suppressed the intracellular luciferase level corresponding to HCV RNA in a dose-dependent fashion (Fig. 1A). At a dilution of 100-fold, Alpha-zam

completely inhibited HCV replications. In contrast, Alpha-zam hardly affected the number of viable cells at this dilution, indicating that the substance is a selective inhibitor of HCV replication. The HCV protease inhibitor Telaprevir (TLV) was also examined and the anti-HCV activity was found to achieve approximately 80% inhibition of HCV replication at a concentration of 4 μM (Fig. 1A and data not shown). The 50% effective dilution (ED_{50}) and 50% cytotoxic dilution (CD_{50}) of Alpha-zam were 761- and < 100-fold, respectively, in LucNeo#2 cells (Table 1). The selective inhibition of HCV replication by Alpha-zam was also confirmed by real-time RT-PCR in LucNeo#2 (Fig. 1B) and in the full-genome HCV replicon cells NNC#2 (Fig. 1C). In this case, the ED_{50}s of Alpha-zam were 540- and 370-fold in LucNeo#2 and NNC#2 cells, respectively. Again, its CD_{50}s were < 100-fold for both replicon cells.

Since it was possible that Alpha-zam exerted its anti-HCV activity through the induction of interferons in the host cells, Alpha-zam was examined for its ability to induce OAS-2 in LucNeo#2 and NNC#2. The cells treated with interferon-α (100 U/ml) upregulated significantly the mRNA of OAS-2 (data not shown). However, such upregulation was not observed for the cells treated with either TLV (4 μM) or Alpha-zam (from 1,600- to 100-fold dilutions). These results indicate that the anti-HCV activity of Alpha-zam is not due to the induction of interferons. In addition, Alpha-zam was found to be inactive against the replication of HBV (data not shown).

Table 1: Anti-HCV activity of Alpha-zam in LucNeo#2 cells

Compound	ED_{50}[1] (fold-dilution)	CD_{50}[2] (fold-dilution)
Alpha-zam	760.9 ± 119.1	< 100

[1] Fifty percent effective dilution based on the inhibition of intracellular luciferase activity.
[2] Fifty percent cytotoxic dilution based on the viable cell number.
The experiments were repeated three times, and mean ± SD values are shown.

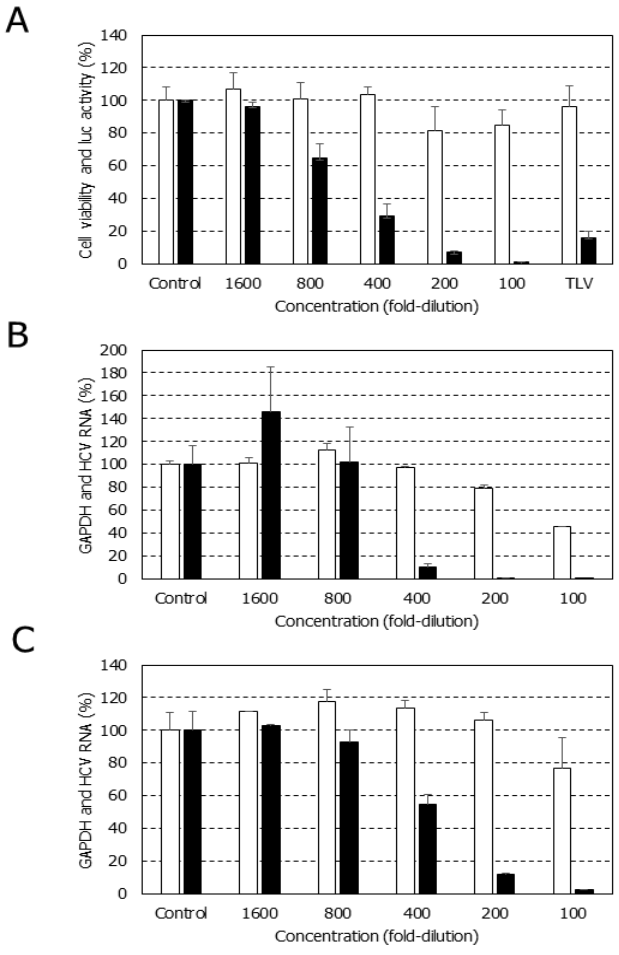

Figure 1: Anti-HCV activity of Alpha-zam in replicon cells.

LucNeo#2 (A and B) and NNC#2 (C) cells were cultured in a 96-well plate in the absence of G418 and in the presence of various dilutions of Alpha-zam. After incubation for 3 days, the number of viable cells was determined by a dye method (open columns). The anti-HCV activity (closed columns) was determined by the inhibition of luciferase activity in LucNeo#2 cells (A) and the inhibition of HCV RNA synthesis in LucNeo#2 (B) and NNC #2 cells (C). As a reference compound, TLV (4 μM) was also examined in LucNeo#2 cells (A). Representative results of repeated experiments are shown.

Discussion

The very high cost of the currently available DAAs has limited its usefulness in combating the deleterious effect of chronic HCV infection. Hence, the need to search for novel compounds from other sources to make treatment available and cheap particularly in the context of low and middle income countries, where larger number of chronic HCV patients who cannot afford the treatment are found. Medicinal plants are valuable reserves for pharmacologically active entities and presently 11% of the 252 drugs considered as basic and essential by the World Health Organization (WHO) are exclusively from flowering plants (Veeresham, 2012). This is because natural products have good safety profiles, low incidence of side effects and also acts at multiple sites, which reduce the potential for resistance. Although there has been domination of synthetic materials for drug production in the last decades, the potential of bioactive constituents of plants for new and novel products for disease treatment is enormous and should be exploited for man's use (Veeresham, 2012).

This study revealed that Alpha-zam, an indigenous formulation containing *Nigella sativa* as its main ingredient, had selective anti-HCV activity. Alpha-zam showed a potent anti-HCV activity in genotype 1b subgenomic and full genome HCV replicon cells. Previous complementary results demonstrating the potency of *Nigella sativa* in the treatment and prevention of HCV-induced complications have been obtained in human and animal studies. A study by Abdel-Moneim (2013) reported the potency of *Nigella sativa* in the treatment of HCV among clinical cases in Egypt. The treatment significantly reduced the viral load and augmented α-fetoprotein and other liver function parameters among the cases. Another study by Barakat et al. (2013) investigated the beneficial effect of *Nigella sativa* oil administration to patients with cirrhosis and chronic liver disease. A significant reduction of viral load and total cure (seronegative status) among 50% and 16.67% of the cases was observed. The study also reported lowering of the blood glucose level, increasing antioxidant activity, and improvement in the general clinical conditions of the patients. Improvement of indices useful for minimizing HCV-induced hematological complications was reported in treated rats (Tekeoglu, 2007). Similar study by Zaher et al. (2008) documented potent anti-inflammatory, antiviral, and antineoplastic activities of *Nigella sativa* in vitro and in vivo, while its major constituent (thymoquinone) was found to be protective to organs including liver from oxidative damage by free radicals (Burits and Bucar, 2000; Nagi and Monsour, 2000; Meral et al., 2001; Mahmoud et al., 2002; Kanter, 2008; Radad et al., 2009; Fouda et al., 2014). The beneficial effect of reducing oxidative stress was further established in animal models with liver ischemia (Yildiz et al., 2008).

In conclusion, this study indicates that Alpha-zam is a selective inhibitor of HCV replication and therefore could be a potential anti-HCV drug. Further studies are on-going to isolate, characterize, and identify the mechanism of action of its bioactive components.

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