

Inhibitory Effects of Thiazolide Compounds against Hepatitis C virus: *in vitro* Study

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Abstract Hepatitis C virus (HCV) is estimated to infect nearly 200 million individuals globally, including approximately 10 million in Pakistan. The newly approved antiviral medicines belonging to Direct Antiviral Agents (DAA) compounds are very expensive and their access is difficult for poor patients. There is a need to develop the more effective, cheap and less toxic antiviral drugs that can be used along with standard PEG-IFN α and Ribavirin therapy that is still in use in developing countries. In this *in vitro* study, we examined the inhibitory effect of Thiazolide derivatives (RM4832 and RM4863) against hepatitis C virus in Huh-7 cell lines. The RNA quantitative results showed a sharp significant antiviral response shown by both of the compounds by decreasing the viral titers after 24 hours of incubation. Compounds showed improved antiviral activity at the dose of 2.0 μ l (400 μ M concentration) and 2.5 μ l (500 μ M concentration) respectively. This study provides the basis for future work on these compounds especially to determine the specific pathway and mechanism for inhibitory action in the replicon systems of viral hepatitis. Thiazolide derivatives show promising inhibitory effects against HCV genotypes 3a infection, which suggests its possible use as complementary and alternative medicine for HCV viral infection.

Keywords HCV, Genotypes, Huh-7 cell line, Thiazolides

1. Background

Hepatitis C virus (HCV) is a blood borne virus and its infection is a main cause of chronic liver diseases [1]. About 170 million people are chronically infected with this deadly virus and almost 500,000 deaths occur annually due to hepatitis C related diseases [2]. Like some of the other developing countries Pakistan has also been recognized as one of the countries of the world where hepatitis C virus (HCV) is endemic [3]. Majority of HCV infections direct to chronic hepatitis, which in turns proceed to liver diseases including fibrosis, cirrhosis and eventually hepatocellular carcinoma (HCC) [4-6]. It is estimated that annually there is a risk of 1-4% among patients to develop hepatocellular carcinoma (HCC) from cirrhosis [7, 8]. HCC is more widespread in patients of age 60 years and older, especially in males [9]. In Pakistan the frequency of HCC ranges from 3.7% to 16% of malignant tumors and the important and most common cause of HCC is viral hepatitis C [10].

Normally HCV has been classified into six major genotypes and their prevalence diverge from one region to another in the world [11]. In Pakistan a strong relationship between chronic HCV infection and genotype 3a and development of HCC has been reported [12]. Although Pakistan has more than 10 million people carrying HCV infection but unfortunately the disease is normally diagnosed at much delayed stage like other under developed and developing countries because of limited health facilities which then leads to chronic infections later on [13].

Treatment of hepatitis C was mainly dependent upon the combination of pegylated interferon alpha (PEG-IFN α) and ribavirin. This treatment was also considered as “standard of care (SOC)” treatment for the past ten years or so. The efficiency of this SOC has been increased by the use of two protease inhibitors mainly telaprevir or boceprevir in hepatitis C genotype 1. In year 2011 FDA (US Food and Drug Administration) approved the use of these protease inhibitors as HCV therapy [14]. Recently some of the approved antiviral medicines with higher sustained virological response (SVR) rates has come into the market for treatment of HCV. These medicines include molecules like sofosbuvir, ledipasvir and simeprevir which belongs to direct antiviral agents (DAA) and can treat HCV within 8-12

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weeks of dosage with SVR up to 90% or greater [15]. In developing countries like Pakistan, where the combination therapy of PEG-IFN α and ribavirin is still in use, although having moderate anti HCV activity and multiple side effects, because the access to newly synthesized and approved drugs is very low [16]. The main side effects of combination PEG-IFN α and ribavirin therapy directs to thyroid dysfunction especially Hypothyroidism [17]. Hematological disorders mainly considerable fall in haemoglobin, platelets count and total leucocyte count in majority of patients at 24 weeks of Interferon plus ribavirin therapy [18] and weight loss were also reported [19]. The resistance of drug owed to viral mutation, high cost and severe side effects make it necessary to explore some other antiviral agents for the treatment of HCV that may strengthen the therapeutic landscape, especially in low income countries. In this regard, several hundreds of plants, pharmaceutical extracts and synthetic compounds has also been used for the treatment of HCV.

Nitazoxanide (NTZ 1), the first and broad spectrum Thiazolide which is considered as anti-infective agent and is approved in the United States for the cure of diarrhea. These small- molecule drugs are also considered as broad-spectrum antiviral agents which target the processes involved in host regulated viral replication [20]. Its effective circulating metabolites such as tizoxanide 2 and some other similar thiazolides are useful for both DNA and RNA viruses [20]. NTZ 1 is an efficient anti-HCV agent in all three conditions whether it is used alone, along with pegylated Interferon or along with combination therapy of both PEG-IFN α and ribavirin [21, 22]. However a major boost occurred in rapid (RVR) and sustained virological response (SVR) if triple therapy of NTZ1, PEG-IFN α and ribavirin used in comparison to combination therapy without NTZ1. If NTZ1 was used alone for 4 weeks and after that pegylated IFN and NTZ1 were used for 36 weeks, without ribavirin, then SVR in 80% of patients was observed [22]. This suggested a guarantee for the treatment of those patients who are incapable to bear ribavirin. These consequences can be compared to direct acting antiviral agent (DAA) such as telaprevir or boceprevir in combination with PEG-IFN α and ribavirin. Recent studies showed that nitazoxanide reduces ATP-sensitive intracellular Ca²⁺ stores which results in slight endoplasmic reticulum (ER) stress by the use of bovine viral diarrhea virus (BVDV) as a surrogate for HCV infection [23]. From these studies, it is believed that both NTZ1/tizoxanide2 and the newer thiazolides, present significant assurance in the treatment of HCV especially for those patients who are incapable to bear ribavirin. These consequences can be compared to direct acting antiviral agent (DAA) such as telaprevir or boceprevir in combination with PEG-IFN α and ribavirin.

Considering the above-mentioned facts, the purpose of present study was to determine the toxicity of selected Thiazolides derivatives in human hepatoma (Huh7) cell lines and to evaluate their antiviral activity in the Huh-7 cell lines infected with HCV serum.

2. Methods

Sample Collection and Cell lines

Sera samples from more than 50 patients chronically infected patients with HCV without any previous history of antiviral treatment were received from Genome Centre for Molecular Diagnostic and Research (GCMD), Lahore, Pakistan. At least 10 Patients of each gender (M/F) were selected with age group of about 20 years to 50 years and with High titer (Viral load) of about 1 million plus of HCV. The participating subjects gave informed consent for the collection of blood samples for this study. The estimated duration of infection varied from 6 months to 15 years and the diagnosis of chronic HCV was based on elevated serum ALT (SGPT) and AST (SGOT) levels at least for six months, histological examination and detection of serum HCV RNA. Children were excluded from the study and all patients were negative for HBs Ag. The Human Hepatoma (HuH-7) cell lines were obtained from Cell bank of Department of Biology, Lahore University for Science & Technology (LUMS), Lahore-Pakistan.

Thiazolides Derivatives

Two Thiazolide derivatives were used in this study. These were dissolved in dimethyl sulfoxide (DMSO) as solvent to make Stock solution and further working dilutions in DMEM. Dilutions ranging from 10 μ mol-100 μ mol concentration were prepared. Two of these compounds RM4832 and RM-4863 were used for the determination of anti-HCV activity *in vitro* using Huh-7 cell lines named as compound A and compound B respectively.

Cell culturing

The Huh-7 cell line is routinely grown in Dulbecco's modified eagle medium (DMEM) supplemented with 100 μ g/ml penicillin, 100 μ g/ml streptomycin, 10% fetal bovine serum at 37°C with 5% CO₂. They are highly permissive for the initiation of HCV replication. The Huh-7 cell line was routinely grown in Dulbecco's modified eagle medium (DMEM) supplemented with 100 μ g/ml penicillin, 100 μ g/ml streptomycin, 10% fetal bovine serum at 37°C with 5% CO₂. They are highly permissive for the initiation of HCV replication. The Huh-7 cell line grown in DMEM was split and plated in 6 wells plate for toxicological analysis of Thiazolide derivatives which has been previously optimized using different dilutions to achieve the maximum inhibitory effects of compound against HCV.

Toxicological Analysis

Huh-7 cells were seeded in DMEM with 10% fetal bovine serum and 1% penicillin and streptomycin at 37°C in an atmosphere of 5% CO₂ in 6 well plate. These cells were treated with different concentrations of compounds of Thiazolide, considering first well as control and added different concentrations of the compound from lowest to highest in the wells. After different intervals the cells were trypsinized and were counted through haemocytometer by using trypan blue dye exclusion method.

Anti HCV analysis of Thiazolides derivatives

For *in vitro* replication of HCV, Huh-7 cell line was used as described previously [24, 25]. For extraction of total RNA from the Huh-7 cells which have been infected with HCV serum Purescript RNA Isolation kit (Life Technologies, USA) was used and HCV REAL-TM Qnat SC kit (Secace Biotechnologies, Italy) was used for Real Time quantification of Extracted HCV RNA according to given protocol.

3. Results

Toxicity analysis of Compounds

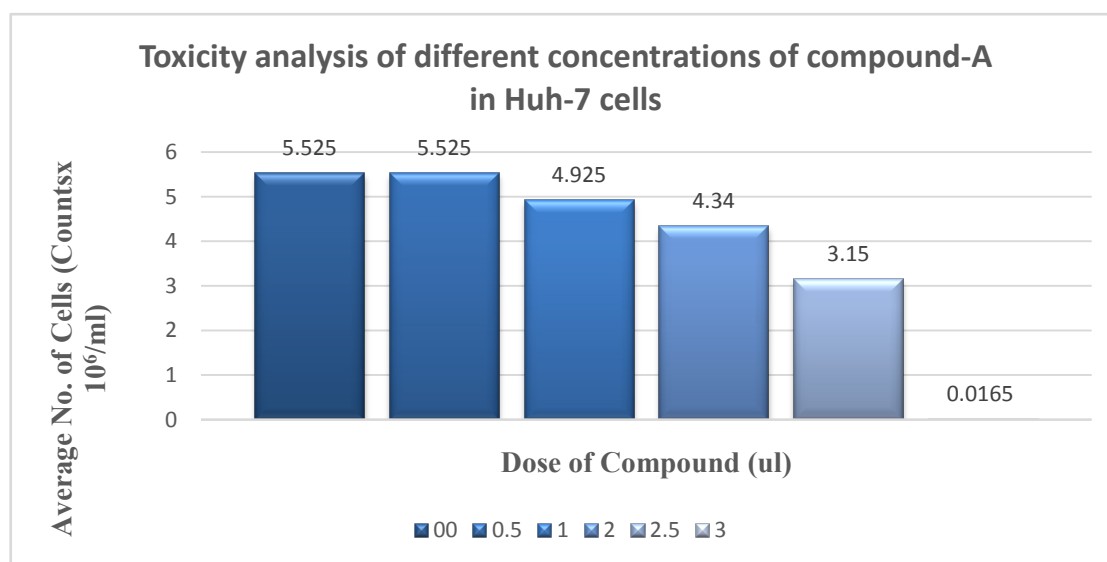
Toxicity of Thiazolide Derivatives were initially determined in Huh-7 Cell Lines. These cells were treated with different concentrations of Thiazolide Derivatives. The viability of Huh-7 cells was confirmed at different concentrations by using Trypan Blue Exclusion Test by using cell suspension and trypan blue dye in ratio of 1:1. Cytotoxicity of compounds A & B were evaluated in Huh-7 cells at different doses ranging from 0.5 to 3.0 μ l in duplicate and their concentration ranging from 100 μ M to 600 μ M respectively. Huh-7 cells were treated with increasing concentrations of Compound A & B for 24 hours and assessed for cytotoxicity by using the cell counting by haemocytometer. The Average number of viable cells per ml in 6 well plates was calculated. It is clear from the results that up to a dose of 2.5 μ l, the compound-A and 2.0 μ l, the compound- B were non-toxic to the cells and were toxic at higher concentration. The concentration at which more than 70-80 percent of the cells survived was considered as non-toxic. 1X-PBS was used as a control. Administrations of up to 2.5 μ l and 2.0 μ l of the drugs A and B were found to be

non-toxic (Figure 1 & 2). At these concentrations cells did not display any significant cytotoxicity during cell passage and trypan blue counting. Cells viability was counted by haemocytometer and trypan blue stain method.

Anti-HCV analysis of Thiazolide Derivatives

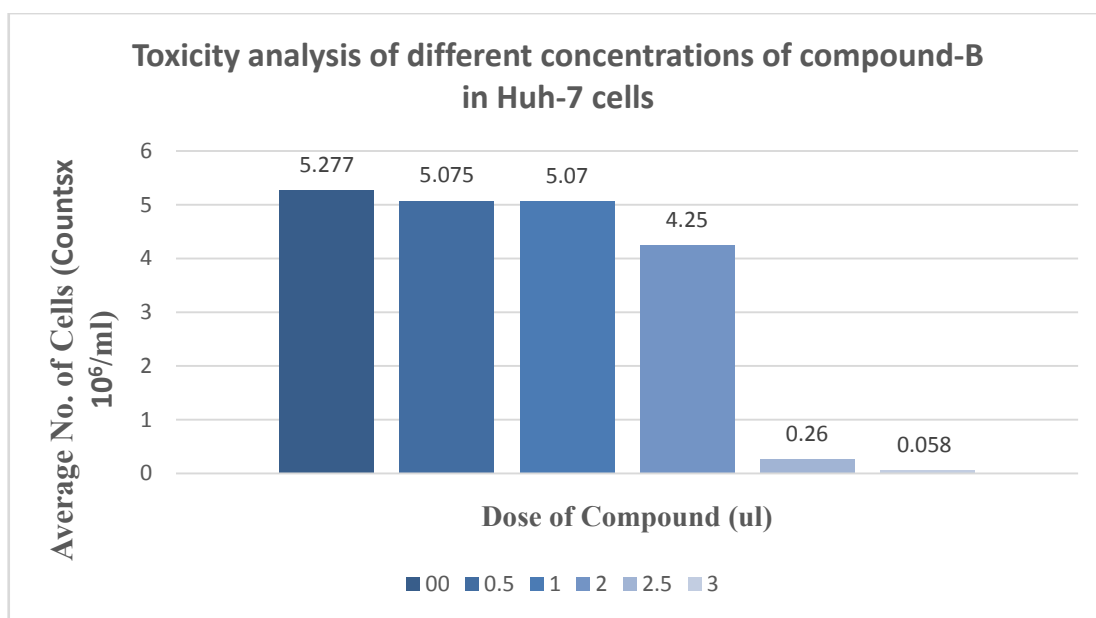
Antiviral activity of compound A& compound B were determined in Huh-7 cells at two different concentrations of 2.0 μ l and 2.5 μ l for both. DMSO was used as Negative control and Positive HCV infected serum sample without any drug was used as positive control. In each 6 well plate one patient sample were treated for both compounds along with Positive control and Negative control. After 24 hours incubation, total RNA was extracted and Real-time PCR was performed for quantitative detection.

The results showed that the antiviral effects of two Thiazolide compounds RM4832 and RM4863 namely A & B respectively, in Huh-7 cell lines. The concentrations of 100-600 μ M with volume of 0.5 to 3.0 μ l of both compounds were used. The non-toxic dose of the compounds was added to cells at the time of infection. The RNA quantitative results showed a sharp significant antiviral response shown by both of the compounds "A & B" decreasing the viral titer of genotype 3a after 24 hours of incubation. Compound "A" showed enhanced antiviral activity at the dose of 2.5 μ l (500 μ M concentration) whereas the Compound "B" showed enhanced antiviral activity at the dose of 2.0 μ l (400 μ M concentration) as compared to the positive control samples in both male and female groups. The present study provides experimental data of two Thiazolide derivatives (RM4832 and RM4863) for clinical treatment of chronic hepatitis C virus infection suggesting that these compounds can be use as complementary and alternative medicine for the treatment of HCV infection (Figure 3 & 4).



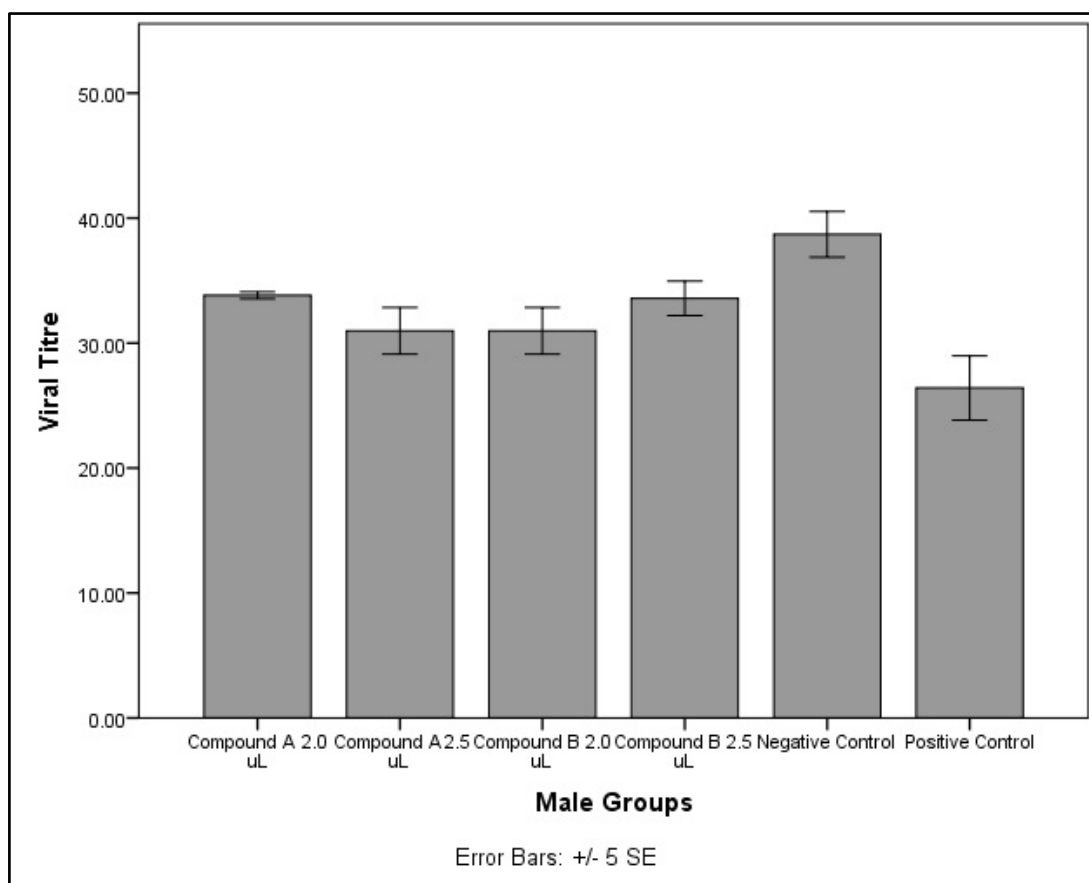
Toxicity analysis of different concentration of Compound-A from 0.5 μ l to 3.0 μ l dosage. Y-axis shows the average number of cell counts x10⁶/ml.

Figure 1. Toxicity analysis of different concentrations of compound-A in Huh-7 cells



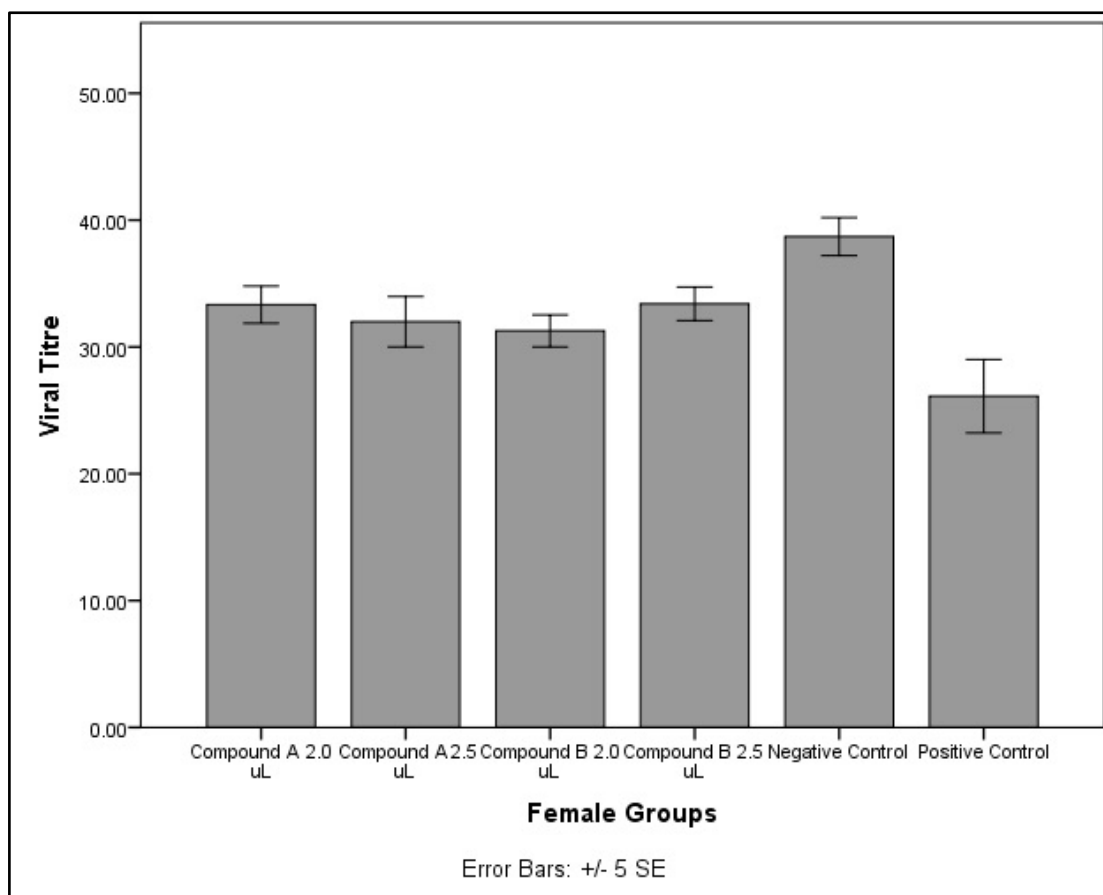
Toxicity analysis of different concentration of Compound-B from 0.5ul to 3.0ul dosage. Y-axis shows the average number of cell counts x10⁶/ml.

Figure 2. Toxicity analysis of different concentrations of compound-B in Huh-7 cells



Antiviral activity of Thiazolide derivatives A & B at 2.0ul and 2.5ul in Male patients. Line 1 & 2 shows Viral titer at 2.0 and 2.5ul of Compound-A and Line 3 & 4 shows viral titer at 2.0 and 2.5ul dose of Compound-B, Line 5 is for Negative control and line 6 shows viral titer of Positive control without any compound.

Figure 3. Antiviral activity of Thiazolide derivatives A & B at 2.0ul and 2.5ul in Male patients



Antiviral activity of Thiazolide derivatives A & B at 2.0ul and 2.5ul in Female patients. Line 1 & 2 shows Viral titer at 2.0 and 2.5ul of Compound-A, Line 3 & 4 shows viral titer at 2.0 and 2.5ul dose of Compound-B, Line 5 is for Negative control and line 6 shows viral titer of Positive control without any compound.

Figure 4. Antiviral activity of Thiazolide derivatives A & B at 2.0ul and 2.5ul in Female patients

4. Discussion

Currently PEG-IFN α in combination with ribavirin is remarkably successful in patients to achieve sustained virological response (SVR) but some severe side effects and high cost of treatment are hurdles to the success of this treatment [26, 27]. Numerous plants and pharmaceutical extracts are also important for the treatment of HCV [28]. Nitazoxanide (NTZ1), an important Thiazolide and its circulating metabolite tizoxanide2 and analogous thiazolides are found to be efficient against both RNA and DNA viruses and a considerable enhancement was observed in Sustained virological response (SVR) as well as Rapid Virological Response when triple therapy of NTZ1/ PEG-IFN α /ribavirin was used in comparison with "SOC" without NTZ1 for the treatment of HCV [22].

The data suggest that two derivatives of Thiazolides (RM4832 and RM4863) could be excellent antiviral agents against HCV. Antiviral effects of NTZ, TIZ and RM4832 against a genotype 1a full length replicon was already reported [29] that were equivalent to those reported for AVA5 cells for genotype 1b [30]. NTZ was an effective inhibitor of an NS5B and two NS3 drug-resistant mutants in

Huh7 cells. No significant differences in potency of NTZ relative to that observed for 'wild type' HCV (in AVA5 cells) was observed for any of the drug-resistant mutants tested. Although the mechanism of action of NTZ is not known, the divergence of the intracellular protozoa and viruses inhibited suggests that a cellular, rather than common viral function is being affected. It is conceivable that changes in the intracellular environment induced by NTZ may alter the effect of subsequent treatment with other anti-HCV agents that also act through the induction of cellular pathways, particularly IFN α . Thiazolides may present the opportunity of new or more efficient combination treatments for chronic HCV, whether it is used as additional agent with currently used SOC treatment or in a substitute for ribavirin, or as an ingredient along with the novel combination course of therapy with other anti-HCV drugs [30].

5. Conclusions

We suggest that there could be excellent therapeutic agents in Thiazolide compounds for the treatment of HCV infections. However, further studies are required to identify

the specific active ingredients by using various techniques. This study also provides basis for future work on these compounds especially derivatives A to determine its mechanism of action in the replicon systems and clinical trials for the treatment of the hepatitis C. Moreover, this study will be helpful to explore the new horizons of the discoveries of the Thiazolide derivatives, which shows promising inhibitory effects against HCV.

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Abbreviation

Pegylated interferon alpha: PEG-IFN α , Hepatitis C virus: HCV, Hepatocellular carcinoma:HCC, Standard of care: SOC, Human Hepatoma: HuH-7, Nitazoxanide: NTZ 1, Sustained virological response: SVR, Dulbecco's modified eagle medium: DMEM.

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