EFFECTS OF SHORT RNA SEQUENCES ANALOGS AGAINST HCV GENOTYPE 4(A) IN REPLICON CELLS

BY

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ABSTRACT

Aim

To determine to what extent short RNA sequences structural analogs could inhibit HCV genotype 4a (ED43) replication in cultured cell with sub-genomic HCV replicons.

Method

HCV short RNA sequences designed to be structurally similar to X region in the 3'-UTR of HCV 1b genome, were inserted into **B*am*H I and H*ind* III** restriction sites in pSilencer 4.1 CMV puro plasmid, transfected into human hepatoma cells Huh 7.5 with subgenomic replicons HCV genotype 4a (ED43/SG.Feo). 48 hrs after decoy transfection HCV RNA were isolated and quantified by real-time Polymerase chain reaction.

Results

the RNA structure analogs 5B46, X12, X12a and X12b were able to inhibit RNA levels by 65.5%, 38.5%, 64.5% and 68% respectively, in HCV genotype 4a.

Conclusion

Despite the sequence dissimilarity of the HCV RNA analogs and HCV genome in the replicon cells, there were enough 3 dimensional structural similarities to inhibit HCV viral replication.

Introduction

Hepatitis C virus (HCV) is a major cause of acute and chronic liver disease, cirrhosis, hepatocellular carcinoma and liver failure, and it remains the leading indicator for liver transplantation **(Lindenbach and Rice, 2013)**. More than 170 million individuals worldwide are infected with HCV, and the cirrhosis and hepatocellular

carcinoma induced by HCV infection are life-threatening diseases **(Shiokawa et al., 2014).**

HCV is an enveloped, positive-strand RNA virus and is the type member of the genus Hepacivirus within the family *Flaviviridae* **(Lindenbach and Rice, 2013)**. The genome encodes a large polyprotein of 3010 amino acids **(Ashfaq et al., 2011)** approximately 9.6 kb in length. The viral genome consists of a single open reading frame (ORF) encoding a polyprotein which is co- and posttranslationally cleaved by host and viral proteases to yield three structural (core, E1, and E2) and seven nonstructural (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins **(Gottwein et al., 2010)**. The ORF is flanked by 2 highly structured untranslated regions (UTRs) that play crucial roles in regulating the translation and replication of the viral genetic material. In addition, a small protein of unknown function is generated from an alternate reading frame as a result of a ribosomal frame shift **(Jackowiak et al., 2014).**

According to a recently updated nomenclature, HCV is classified into 7 genotypes, which are subdivided into 67 epidemiologically diverse subtypes. The distinction between individual genotypes and subtypes has often been expressed as the proportional difference in their full genome nucleotide sequences. Accordingly, the HCV genotypes differ from each other by approximately 30%, and the subtypes differ from each other by at least 15 % **(Ashfaq et al., 2011).**

HCV strains from different parts of the world show significant genetic heterogeneity, and on the basis of phylogenetic analysis, HCV has been classified into seven genotypes and a number of subtypes **(Saeed et al., 2012)**. Because different HCV genotypes differ in their biology, as well as in their sensitivity to therapeutics and neutralizing antibodies, it is of great importance to create research tools for all major genotypes. Thus, it is essential to have functional cDNA clones representing the major HCV genotypes and important subtypes **(Gottwein et al., 2010).**

The recent development of subgenomic and full-length HCV replicons that replicate and express HCV proteins in stably transfected human hepatoma cell-derived Huh-7 cells has facilitated the analysis of the role of cellular pathways required in HCV replication and the efficacy of antiviral drugs **(Kapadia et al., 2007).**

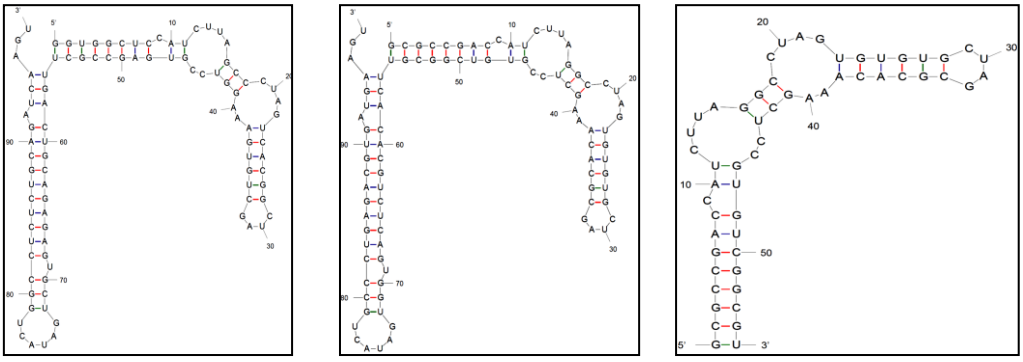
The HCV replicons have made it possible to determine the roles of viral proteins and RNA sequence and structures in HCV RNA replication **(Cai et al., 2007).** When *in*

vitro-transcribed RNA from such constructs is introduced into Huh-7 or its highly permissive sublines (e.g., Huh-7.5) and selected with G418, replicon-containing cell clones can be generated with persistently replicating HCV RNA. These replicons have been very useful in understanding the viral and host factors involved in HCV replication as well as for screening and evaluation of replication inhibitors **(Saeed et al., 2012).** Moreover, subgenomic replicons contain all prime targets for antiviral therapy, i.e., the NS3 protease and the NS5B RdRp, and therefore provide a suitable platform for cell based screenings **(Lohmann and Bartenschlager, 2014).**

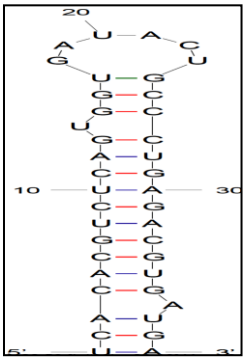
In the present study, we observe the effect of short RNA sequences analogs having structural similarity to HCV genome, on the HCV viral replication in cell replicon of HCV genotype 4a (ED43).

Materials and Methods

HCV RNA structure analogs



A **b** **c**



d

Figure (1): RNA
structural analogs similar to the X region of HCV genotype 1b. (a.X94, b.X12, c. X12a, d.X12b)

HCV RNA sequences, X12 was constructed to be 59% identical to an RNA sequence X94 which has 100% sequence similarity to the X region (9508-9605 nts in the 3' –

UTR end of HCV genotype 1b. HCV RNA structure analogs, X12a and X12b were designed to be approximately similar to X94, but were predicted to have the same secondary structure, figure (1), and using web server Mfold ver. 3.2. An unrelated hepatitis B sequence (HB analogos), was used as the negative control.

Decoys	Genotype 4 (ED43)
X94	99%
X12	No sig. similarity
X12a	No sig. similarity
X12b	No sig. similarity
HB	No sig. similarity
5B46	No sig. similarity

Table (1): Analogues sequence similarities

Expression Plasmids for HCV analogs:

DNA fragments containing the sequence analogs were inserted into the *BamHI* and *Hind III* sites of pSilencer 4.1CMV puro plasmid. Plasmids were purified using **(Endofree® Plasmid Purification Maxi Kit, Qiagen)** according to manufacturer's instructions.

Replicon cells Human hepatoma cell line (Huh 7.5), containing bicistronic subgenomic replicon of HCV genotype 4a (ED43/SG Feo) were used. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) enriched with 10 % fetal bovine serum (FBS) and 500 µg/ml G418.

Cell Viability Test (MTT Test) Our (Huh 7.5) cell replicon was plated 2 days before transfection, the cells were transfected and MTT assay was done after 48 hours from transfection.

Transfection of the cells with the HCV RNA structure analogs The Huh 7.5 cells carrying the HCV RNA genotype 4a were seeded in 12 well plates and grown till 75% confluence. Cells were transfected with 16 µg of DNA plasmid containing the HCV RNA structure analogs with 16µl Lipofectamine 2000 *(Invitrogen).* For transfection plasmid-Lipofectamine complexes were formed in two stepped process. In step 1, 16µl Lipofectamine 2000 was diluted with 800 µl of Opti-MEM I Reduced Serum Medium (Invitrogen) and incubated for 30 min. In a separate tube 64 µg plasmid DNA was added to 800 µl of Opti-MEM I Reduced Serum Medium (Invitrogen) plus 16 µl of **Plus ™ reagent *(Invitrogen)*** to enhance transfection, incubated for 15 min. In step 2 preincubated Lipofectamine solutions were added to plasmid DNA mix. Lipofectamine-plasmid complexes incubated for 30 min before adding to replicon cells. After 6 h of incubation at 37^{o}C, 1ml of growth media (DMEM) with 10% FBS were added, and allowed to grow for another 48 h. HCV RNA levels of the cells were determined using quantitative real time PCR.

Quantitative Real Time PCR

RNA was isolated from the cell replicons with the RNeasy® Mini Kit, Qiagen, and treated with 1µl (1U/ µl) DNase I, amplification grade (Invitrogen). The concentration of RNA was measured by Nanodrop (ND1000-Spectrophtometer, *Thermo Scientific*). 2µg of RNA was reversed transcribed using the Superscript® III First Strand Synthesis System for RT-PCR (Invitrogen). The concentration of resulting cDNA was determined by RT-PCR using the Power Syber® Green according to the manufacture's protocol (Life Technology).

Specific primers for HCV genotype 4a (GU814266.1) forward primer 5'CTACAACCGTTCCCCAGGAC3', reverse primer 5'CATCGTAGCACTCGCAAAGC3' were used in real time PCR. As internal control (House Keeping gene) the level of lactate dehydrogenase (LDHA) expression was measured and used to normalize HCV RNA level in the cells. The primers sequence for LDHA forward primer: 5'TAATGAAGGACTTGGCAGATGAACT3', reverse primer: 5'ACGGCTTTCTCCCTCTTGCT3'.

Assays were performed in triplicate, and the results were expressed as mean ±SE of HCV replication as a percent of untreated (lipofectamine alone) control. The Student's *t*-test was used to evaluate comparisons and $p<0.05$ was considered to indicate a significant difference.

Results

The RNA analogs used in the transfection of cell replicon of HCV genotype 2a, 3a and 4a, were derived from an RNA sequence (X94), which have 100% sequence similarity with the X region (95408-9605 nts) in the 3'- end of HCV genotype (1b), to have similar secondary structure to the HCV genome but with different sequence. The inhibition of viral replication of HCV was assessed by quantifying the RNA level in the cell replicon using Real-time PCR

Cell viability (MTT Test)

The effect Lipofectin reagent on the cell viability is very important as the reagent is normally toxic for all type of the cells. There was no toxic effect of Lipofectin on the

HCV genotype 4a cell viability, as shown in figure (2), which indicates non-significant changes between the cells treated with Lipofectin and DNA and Cells treated with Lipofectin only and the non-treated cells.

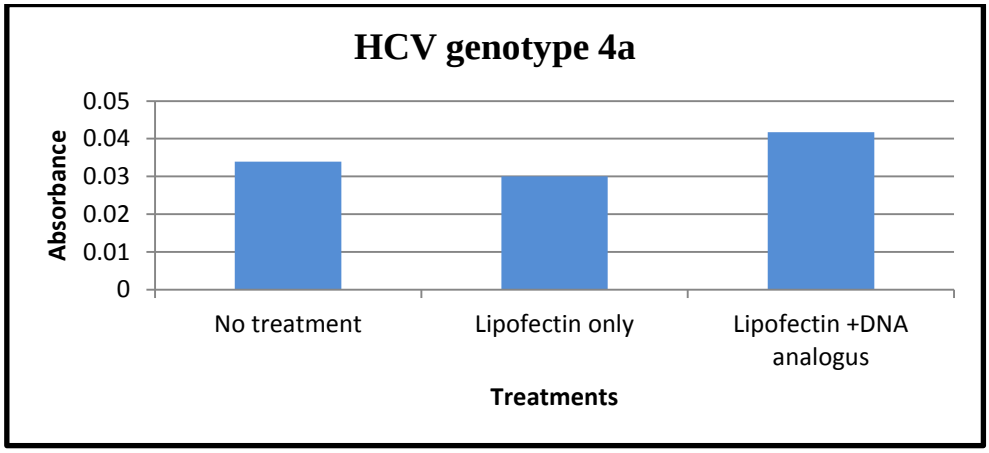


Figure (2): Lipofectin effect on HCV genotype 4a (ED43/SG Feo) cells

Real-time PCR results

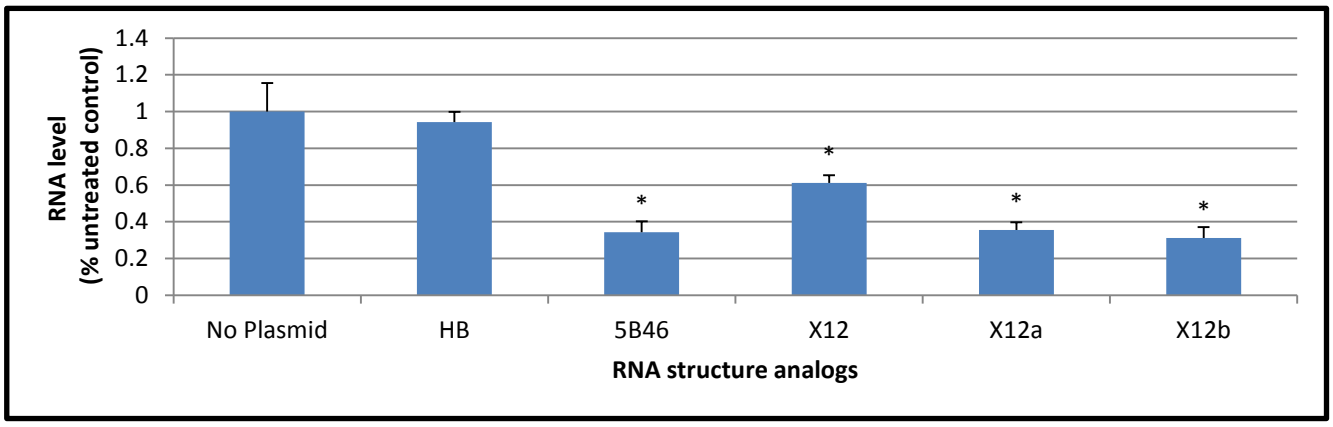


Figure (3): HCV RNA level of HCV Genotype 4 (ED43) replicon cells, 48 hr post transfection with RNA structure analogs. The columns and bars represent the mean ± SE. Statistically significant differences relative to controls are indicated by ($P < 0.05$)

Figure (3) showed that the unrelated HB analogs had no inhibitory effect on the HCV RNA level in all three HCV genotypes (ED43) replicon cells, compared to the cells treated with Lipofectamine alone. Concerning the transfected ED43 cells, figure (4) shows the inhibitory effect of the 5B46, X12, X12a and X12b by 65.5%, 39%, 64.5% and 69%, respectively, compared to the untreated cells and by 63.5%,35%, 62.5% and 66%, compared to the HB analogs at $P<0.05$.

Discussion

Development of new therapeutic approach against HCV is the goal of many recent studies. For several reasons the subgenomic replicon system played a major role in development of HCV-targeting DAAs **(Lohmann and Bartenschlager, 2014)**. Efforts were subsequently focused on interfering with highly conserved genomic

regions. The great advances in the antisense and small interfering siRNA technology prompted the idea of an efficacious therapy in the near future, but in many cases the emergence of resistant viral genomes has been observed for HCV and other viral infections **(López et al., 2007)**. It has been shown that the terminal UTR RNA sequences and structures are relatively well conserved phylogenetically. This stringency of conservation makes the UTRs particularly attractive antiviral targets, in that a single inhibitor may be effective against multiple, if not all genotypes **(Smith and Wu, 2003)**. In the current study, RNA structure analogs to the X region of the 3'UTR end HCV genotype (1b) were able to inhibit the viral replication in the cell replicons of genotype (4a), this is in good agreement with the fact that The 5'UTR and the extreme end of the 3'UTR are the most conserved regions of HCV RNA in terms of primary sequence and secondary structures together with the fact that these structured domains are located at the 5' and 3' ends of the genome, it stands to reason that they play important roles in viral RNA translation and/or replication **(Shi and Lai, 2006)**. HCV RNA has a number of cis-acting replication elements (CREs) whose function could potentially be inhibited by structural RNA mimics. CREs was bearded in the positive-strand NS5B and X region as well as in the negative strand 3'-terminal region. The function of each CRE is assumed to depend on the ability of these structures to bind host factors and viral nonstructural **(Smolic et al., 2010)**. Our study revealed significant inhibition in HCV viral replication in the genotypes 4a cell replicon, when transfected with X12 analogs which have 59% sequence similarity to RNA X94 analogs which are 100% sequence similar to the X region of the 3'-UTR end of genotype (1b) and with no significant sequence similarity to HCV genome of the 3 genotypes, this observation are in line with that the X region forms three stable stem-loop structures that are highly conserved across all genotypes the 3'UTR sequence, particularly the X region, is involved in the regulation of translation **(Shi and Lai, 2006)**.

One of the most promising approaches is the specific recognition of highly structured regions in the genomic RNA that importantly retain a conserved sequence and structure and fulfill essential roles in viral translation and replication. They interact with viral and host proteins to carry out these essential functions for the viral maintenance and propagation. This means that the preservation of both primary and secondary structure in those RNA domains is crucial for the conservation of the function. It has been shown that mutations that preserve the secondary structure of the IRES pseudoknot also result in a drastic reduction of the in vitro translation efficiency. This is in good agreement with the high sequence conservation observed in this domain among different HCV isolates **(López et al., 2007)**.

Previous studies have shown that CREs could potentially be inhibited by structural RNA mimics. Structural mimics based on the HCV IRES were recently shown to inhibit HCV translation *in vitro* and in replicon models **(Smolic et al., 2010)**. We have found in our study, that X12a and X12b analogs which were predicted to have the same secondary structure the native HCV genome, were able to decrease the viral replication in the different HCV genotypes used, which indicate that the activity of the HCV IRES being highly structure dependent, only mutational events that can alter the IRES structure would allow the virus to circumvent inhibition by this approach and as binding of the cellular proteins is probably dependent on the RNA structure, stabler derivatives and small molecule structural analogs of the RNA could be utilized **(Ray and Das, 2004).**

In this study, we have shown that 5B46 structure analogs which was constructed by base pair changes in the stem loop region, without changing the secondary structure, of the 5B74 analogs which was identical to the mimic (NS5B) **(Smolic et al., 2010)**, was able to inhibit HCV viral replication. The Stem Loop (SL) structures in the NS5B coding region were recently demonstrated to function as CRE, and moreover the mutation in SL domain which abrogated its binding to RdRp has also been found to abolish the replication ability of replicon, suggesting that HCV RNA replication likely involves physical interaction between RdRp and SL domain containing CRE **(Zhang et al., 2005)**.

RNA structure analogs were able to inhibit viral replication in the HCV genotype 4a. Our data reflects the importance of the secondary structure of the HCV genome, rather than the primary structure (sequence) in decreasing the viral replication in cell replicon.

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الملخص العربي

تأثار التسلسلات المتماثلة للحمض النووي الريبي ضد فيروس التهاب الكبد الوبائي C الوراثي الرابع في خلاية الربليكون

للسادة الدكاترة

١ اسماعيل محمد الشافعي ٢ نهى مصطفي مصباح ٣ دينا محمد ابوالمعاطي ٤ لمياء نبيل حماد

مـــــــــــــــــن

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يعد فيروس الأتهاب الكبدى C هو السبب الرئيسي للأتهاب الكبدى المزمن و الحاد، حيث أنه يؤدي إلى تليف الكبد وسرطان الكبد. علاوة على ذلك، يعد الفيروس هو السبب الرئيسي لزراعة الكبد. وكان معظم المرضى المصابين بالفيروس لا يدركون إصابتهم، وبالتالي فإنهم لا يحصلون على الرعاية الطبية اللازمة للعلاج من الفيروس. منما يؤدي الى خلق مشاكل اقتصاديه ضخمه في جميع أنحاء العالم، تتعلق معظمها بمعالجة المضاعفات الناتجه من الأصابه بالفيروس بما في ذلك تليف الكبد وسرطنة الخلايا الكبديه. كان لتطور البيولوجى للجين الخاص بفيروس الكبد C، دوراً و تأثيراً كبيراً على الأبحاث الخاصه بالفيروس و علاجه.

ان RdRp هو الأنزيم الأساسي الذى يزيد من معدل تكرار الجينوم الفيروسي. ان البنية والوظيفة الخاصه المنطقه المحفزه للأنزيم محفوظه بين الأنماط الجينية. ويحتاج هذا الأنزيم الى هيكل ثانوى مستقر لأتمام التفاعل المطلوب منه.

في هذه الدراسه، استخدمنا تسلسلات قصيره للحمض النووي الريبي ، X12، X12a وX12b، مصممة لتكون مطابقة للهيكل الثانوي في المنطقة X في المنطقه الغير مترجمه لفيروث ألتهاب الكبد الوبائى نمط 1B الوراثي، فضلا عن عناصر قصيره من stem loop للمنطقه X و التى تم إدراجه إلى البلازميد، الذى يحمل حمض نووي ريبى خداعى.

الهدف من البحث:

الهدف من الدراسة الحالية هو تحديد مدى تنبأ الكمبيوترلتسلسل الحمض النووي الريبي القصير المتشابه مع الهيكل الثانوي للحمض النووي الريبى للفيروس، وتأثيرها على تثبيط تكاثر الفيروس فى خلايا ريبليكون وتأثيرها على الأنماط المختلفه للألتهاب الكبد الوبائى C (النمط٤).

وتوصل البحث إلى النتائج التالية:

- Lipofectamine المستخدم فى أصابة الخلايا ليس له تأثير سلبى على جميع الخلايا المستخدمه.
- جميع نظائر الهايكل الثانوى كان لها تثبط على خلايا الفيروث.