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Research Article

IN VITRO VALIDATION OF SIRNA ANTAGONISTIC TO HUMAN VCAM -1 IN U937 CELLS

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ABSTRACT

Adhesion of leukocytes endothelial cells of arteries and their migration into the vessel wall is the critical event in initiation of atherosclerosis. This process is caused by the up regulation of adhesion molecules on endothelial cells and an increased expression of chemotactic factors to monocytes, in the vascular wall. Gene silencing by small interfering RNA (siRNA) has emerged as a useful technology for assessment of gene function and study of gene-gene interactions. The active agent, short interfering RNA (siRNA), are known to vary in silencing capacity depending on the position in the mRNA target. The validation of designed siRNA molecules as to their silencing efficiency is carried out in vitro cell based assay. In present study U937 cell line is used as a model as they are differentiating monocytes that express VCAM-11. The designing of perfect siRNA is a crucial step and involves the use of various bioinformatics tools. Presents study aimed at designing and in vitro validation of human VCAM-1 siRNA by qPCR and assessment of their immunotoxicity effect in U937 cells.

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INTRODUCTION

Atherosclerosis is a disease of thickening of arterial wall, due to accumulation of fatty material, cellular waste products, calcium, cholesterol etc. It is mainly due to chronic inflammatory response because of which buildup of white blood cells (WBCs), macrophages, Low density lipoproteins (LDL) cholesterol, fat, calcium etc. occurs in the artery. This buildup is known as plaque, that recruits more WBCs, which further damage the artery and allow more LDL to penetrate the arterial wall. Ultimately, artery becomes inflamed and plaque causes the arterial muscle cells to enlarge and form hard cover. This causes narrowing of the artery reducing the blood flow and increasing blood pressure [1].

Injury to the arterial wall and inflammation is considered to be an important part in the development of atherosclerosis. Researchers have uncovered evidence that atherosclerosis begins with a local injury to the endothelial cells. This injury attracts WBCs, which provoke a natural inflammatory immune response. Inflammation is central to the natural healing process in human body. However, in this case, the inflammation occurs in an inappropriate area and intensity which tends to damage the endothelial layer [2]. The disease is the major cause of death worldwide in developed and developing countries including India and according to world health organization (WHO) fact sheet 2016, it is chief contributor to the cardiovascular disease and accounts for nearly 31% of global death annually and out of which 80% of deaths are due to heart attacks and stroke.

Leukocyte adhesion on activated vascular endothelial cells and their migration into the vessel wall is the critical event in the initiation of atherosclerosis. It has been demonstrated that oxidized low density lipoproteins (ox-LDLs), beta-very low density lipoprotein (β-VLDL) and lipoproteins play an important role during the initial phase of inflammation. Whereas high density lipoproteins (HDLs) are anti-inflammatory in nature [3]. Certain inflammatory pathways molecules, such as P- selectin, intercellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1) are involved in leukocyte rolling, adhesion and transmigration into subendothelial space [4,5].

One such molecule is VCAM-1 (CD106) which is a member of immunoglobulin superfamily and has essential role in the recruitment and adhesion of lymohocytes, monocytes, macrophages, at the site of inflammation [6]. Unlike ICAM -1, VCAM-1 is not expressed in normal condition but it is ready expressed in pro-atherosclerotic conditions [7,8,9]. So, silencing the gene VCAM-1 gene, can be helpful in inhibiting the progression of the disease. Thus silencing molecules can open up newer targets for drug development.

RNA interference (RNAi) technology is based on the natural process of micro RNA (miRNA) post transcriptional gene regulation. The technology involves deploying double stranded small interfering RNA (siRNA) to specifically inhibit gene

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expression by degradation of specific mRNA. Some siRNAs have been reported to elicit immune response in the form of enhanced TLR7 production [10,11,12]. Present study aimed at *in silco* designing and validation of siRNA molecules antagonistic to VCAM-1 along with evaluation of their immuno- cytotoxic effect if any and validation of siRNAs in model cell based assay system.

MATERIALS AND METHODS

Designing of siRNAs

The sequence of VCAM-1 (Gene ID 7412) was retrieved from NCBI data base and three freely available online web servers (siSearch (http://sisearch.cgb.ki.se/); MWG (http://www.mwg-biotech.com/html/) and siDirect (http://design.RNAi.jp/) were used to get potent siRNA molecules. Various designing parameters like GC content of 30-55%, Reynolds score of more than 6, minimum blast hits for minimum off target effects, accessibility to the target mRNA sequence, Hydrogen bond index value as described by Uei-tei and Reynolds [13,14,] were kept in view while designing siRNAs.

In silico Validation

For *in silico* validation of predicted siRNA molecules and their negative control scramble siRNA molecule (designed by scrambling the nucleotide sequence of the siRNA using InvivoGen scramble siRNA Wizard v3.1) for off target effects was carried out; using BLAST programme available at NCBI. Further *in silico* validation of siRNA involved secondary structure prediction of targeted mRNA of VCAM-1 using Mfold software [15] and prediction of the accessibility of the mRNA site for designed siRNA binding.

Growth and viability of U937 Cell Line

U937 cells were procured from National Centre for Cell Science (NCCS), Pune, India and maintained in RPMI 1640 medium containing 10% heat inactivated Fetal Bovine serum and 1% antibiotic solution (penicillin and streptomycin) at 37 C in a humidified atmosphere of 5% CO_2. U937 Cell line has originated from histiocytic lymphoma which displays many monocytic characteristics and is thus used as model for the differentiation of monocytes and macrophage *in vitro* [16]. In present study the cell line has been used since it is known to express VCAM-1 under above mentioned growth conditions.

siRNA synthesis

siRNAs were synthesized by In vitro transcription (IVT) method [17]. siRNAs were transfected into U937 cells at different concentrations using Hiperfect transfection reagent as per manufacturer's protocol along with scrambled siRNAs. All cells were cultured as mentioned above and transfection was carried out in 48 hour old cultures. The harvesting of cells for expression studies was carried out after 24 hours of transfection along with the untransfected controls. The total RNA was extracted from the *in vitro* grown cells using Macherey-Nagel NucleoSpin® RNA II kit as per manufacture's protocol.

Estimation of siRNA silencing Efficiency

The isolated RNA was first reverse transcribed into its complementary DNA (cDNA) using the enzyme reverse transcriptase and oligo dT primer (Fermentas, USA). The resulting cDNA is amplified using VCAM-1 specific primers (Fwd.- GAAGTCCCTGGAAACCAAGAG; Rev.- CCTTCTCAGTCCAAATCCAGTG) in a Thermocycler (Techne, USA). The thermal profile for the reaction is 95 C for 5min, followed by 35 cycles of 95 C for 10s, 55 C for 15s, 72 C for 20s and final extension of 72 C for 10 min followed by storage of samples at 4 C. The band intensities of PCR products were then measured by Gel Doc 1000 (Bio-Rad, USA) and percentage inhibition was calculated.

Out of the three siRNAs, the one giving the maximum inhibition was used to assess the silencing efficiency by Real Time PCR (Eppendorf Master Cycler epRealplex), and relative quantitation with respect to housekeeping gene GAPDH was calculated. qPCR allowed the determination of VCAM-1 expression and percentage silencing in U937 cells.

Immuno-toxicity Assessment

Further immunotoxicity of the validated siRNA was assessed by checking the TLR 7 response in siRNA transfected cells by relative $2^{\Delta\Delta CT}$ quantitaion method [18].

RESULTS

Synthesis and Analysis of siRNAs

Initially the nucleotide sequence of human VCAM-1 gene was used to obtain siRNA sequences using *in silico* design tools. Three *in silico* validated siRNAs were finally selected for synthesis as shown in **Table 1**.

The 19 bp nucleotide sequence of siRNA along with two overhang nucleotide were synthesized by IVT and qualitatively analysed on 20% polyacrylamide gel run at 100 V as shown in **Figure 1**

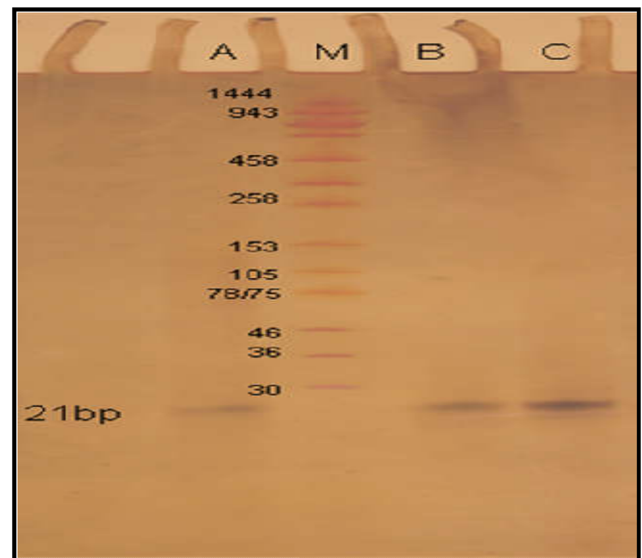

Figure 1 The gel picture depicting siRNA synthesis through In Vitro transcription

U937 Cell Line Culture

U937 cell growth was checked using haemocytometer and it was observed to be maximum at 72 hours interval as shown in **Figure 2**. At that time the cells were in confluence and hence transfection experiments were conducted at 72 hours.

Table 1 List of selected potent siRNAs molecules using various web Tools.

siRNA	Position	siRNA sequence	GC%	Blast Hit	Reynolds Score	H-bond index
A	1983	TT| GACATTCATATACTCCCGC	42.0%	0	7	8
B	1770	TT| GATAATTAATTCCACTTCC	36.8%	0	6	8
C	909	TT| TTGAACAATTAATTCCACC	32.6%	0	6	13

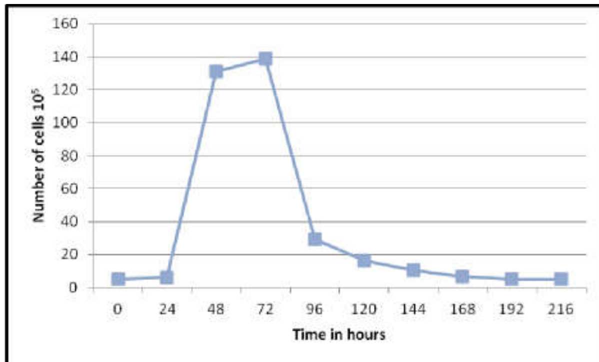

Figure 2 Growth profile of U937 cells

The U937 cells were transfected with 5nM, 10nM and 15nM of siRNA concentrations. The isolated total RNA was reverese transcribed to cDNA. Before cDNA preparation the quality of isolated RNA was checked on 2% Agarose gel as shown in **Figure 3** and two bands of rRNA 28S and 18S were visible in gel.

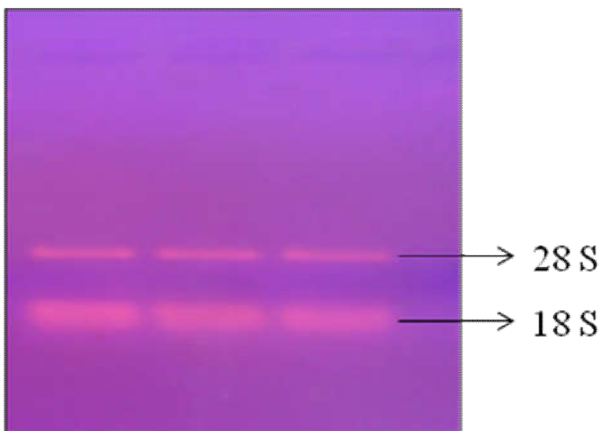

Figure 3 RNA isolated from *in vitro* grown cells.

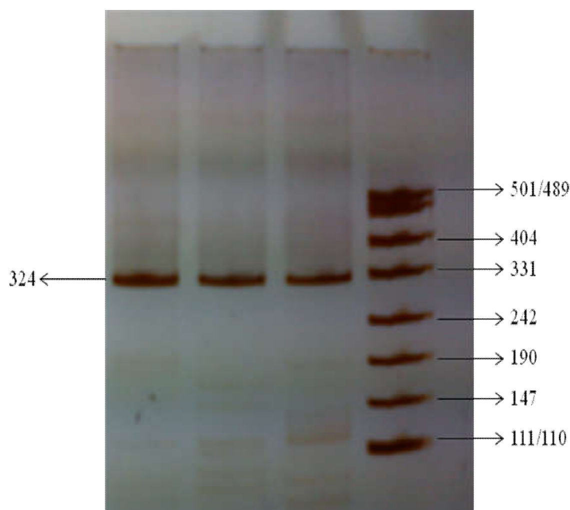

Figure 4 10% PAGE showng PCR Product of 324 bp

On the transcribed cDNA gene specific PCR was carried out which produced a 324 bp product.

The product was qualitatively assessed on 10% acrylamide gel and qunatitative analysis was carried out using band intensities measurements with Gel Doc Quantity One software [19].

Table 2 Silencing efficiency of different siRNAs at various concentrations

Concentration	siRNA A	siRNA B	siRNA C
5nM	67.98%	51.34%	44.33%
10nM	72.89%	58.50%	52.90%
15nM	75.65%	59.12%	56.26%

As shown in **Table 2**, the siRNA A showed the maximum inhibition of gene expression at 15nm concentration and its further validation in Real time PCR through relative quantitation (**Table 3**) shows the silencing efficiency of 91.97% at 15nM of siRNA concentration.

Table 3 qPCR quantification data for VCAM-1 expression in U937 transfected cells

	Control	Hkg	ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$	Gene Expression
Untransfected	19.99	19.98	0.01	0	1	100
siRNA 5nM	21.77	20.64	1.13	1.12	0.4601	46.01
siRNA 10nM	22.1	19.97	2.13	2.12	0.2301	23.01
siRNA 15nM	24.37	20.72	3.65	3.64	0.0803	8.03
Scramble 5nM	20.66	20.57	0.09	0.08	0.9461	94.61
Scramble 10nM	20.21	20.11	0.1	0.09	0.9395	93.95
Scramble 15nM	20.66	20.53	0.13	0.12	0.9201	92.01

The fold change in expression of the VCAM-1 gene normalized to internal control was analyzed using *C*t values provided by Real-Time PCR and only 8% of VCAM-1 gene expression was observed at 15nM. The percentage inhibition was calculated by analyzing values from **Table 3** and the percentage silencing efficiency of 91.97% was achieved at 15nM concentration.

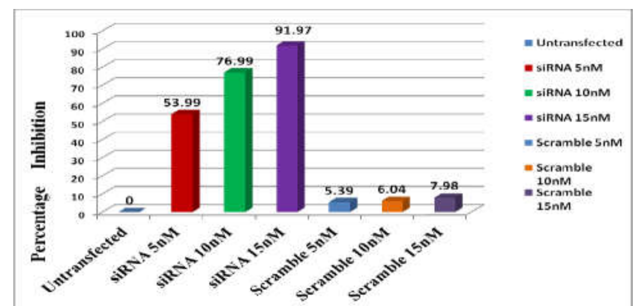

Figure 5 shows the percentage VCAM-1 inhibition at different siRNA concentrations along with scramble siRNA control.

The immunotoxicity effect of siRNA was also accessed and the results obtained (**Table 4**) showed that siRNA molecule do not elicit any immunocytotoxicity effect.

The gene expression values from the **Table 4,** showed that there is negligible change in expression with respect to positive control untransfected sample and that of negative control scramble siRNA.

Table 4 qPCR data analysis for TLR7 response of siRNA

	Control	Hkg	ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$	Gene Expression
Untransfected	19.99	19.98	0.01	0	1	100
siRNA 5nM	19.79	19.73	0.06	0.05	0.9659	96.59
siRNA 10nM	19.83	19.78	0.05	0.04	0.9726	97.26
siRNA 15nM	19.77	19.72	0.05	0.04	0.9726	97.26
Scramble 5nM	19.99	19.95	0.04	0.03	0.9794	97.94
Scramble 10nM	19.86	19.81	0.05	0.04	0.9726	97.26
Scramble 15nM	19.78	19.74	0.04	0.03	0.9794	97.94

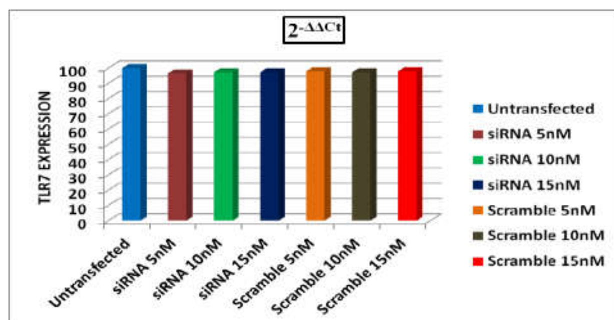

Figure 6 showed the TLR 7 expression in response to siRNA.

Real time data analysis showed the expression of TLR7 gene with respect to siRNA at different concentrations and that of untransfected control are equivalent (**Figure 6**). So this proves that siRNA do not elicit any immune response and TLR7 expression is same as that of control sample.

DISCUSSION

Out of the three *in silico* predicted siRNA molecules, siRNA A has the highest GC content of 42% as compare to siRNA B and C which has GC content of 36.8% and 32.6% respectively. The Reynolds score value of siRNA A is also highest that is 7 whereas that for siRNA B and C the score value is 6. The hydrogen bond index of siRNA A is equal to that of siRNA B that is 8 but is lower than that of siRNA C that is 13. Since H-bond index predicts the bonding between siRNA molecule and that of its target mRNA and silencing efficient is inversely proportional to the H- index value, it was presumed that the siRNA A can knock down the gene expression more efficiently than other two siRNA molecules.

The experimental validation of *in silco* designed siRNA showed that the silencing efficiency of siRNA A that is 75.65% is higher than that of siRNA B and siRNA C that is 59.12% and 56.26% respectively. Further validation via qPCR shows that the silencing efficiency of siRNA A comes out to be 91.97% at 15nM concentration. There have been controversies regarding optimal siRNA concentration for efficient knockdown of gene expression.

70% silencing efficiency was found in commercially available siRNAs from Samchully Pharm. Co., Ltd. (Siheung, Korea) against Green fluorescent protein at 80nM of concentration [20] and silencing efficiency of siRNA against CXCR4 gene was 50% at 50nM concentration [21]. siRNA against 3T3-L1 adipocytes from Dharmacon at 100nM concentration was able to inhibit 70% gene expression. Li and co-workers observed the silencing of 70% at 50nM concentration with anti-survivin siRNA [22]. Comparatively the siRNA designed in present study showed that 91.97% of silencing was achieved at only 15nM of siRNA concentration without eliciting any immune response.

CONCLUSION

So the study concluded that siRNA molecule designed against human gene VCAM-1, are efficient to knock down the gene expression at very low concentration of 15nM and have the silencing potential of 91.97% that too without any immuno-cytotoxicity response. These can be further studied for therapeutic application.

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Web links

1. http://sisearch.cgb.ki.se/
2. http://www.mwg-biotech.com/html/
3. http://design.RNAi.jp/
4. http://www.ncbi.nlm.nih.gov/BLAST
5. http://www.invivogen.com/sirnawizard/scrambled.php

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