miR-33 inhibition attenuates the effect of liver X receptor agonist T0901317 on expression of liver X receptor alpha in mice liver

Abbas Mohammadi[(1)], Hossein Fallah[(2)], Beydolah Shahouzehi[(3)], Hamid Najafipour[(4)]

Original Article

Abstract

BACKGROUND: microRNAs play pivotal roles in metabolism and other aspects of cell biology. microRNA-33 and liver X receptor (LXR) affect lipid metabolism and cholesterol trafficking. In this study, we evaluated effects of co-administration of miR-33 inhibitor and LXR activator on LXR-α and adenosine triphosphate-binding cassette transporter A1 (ABCA1) expression in mice liver.

METHODS: Twenty-four mice were randomly allocated into four groups (n = 6). Group 1 mice received standard chow diet without any treatment, group 2 received 30 mg/kg/48 hour LXR agonist (T0901317), group 3 received 1 mg/kg/48 hour in vivo locked nucleic acids (LNA) anti-miR-33 and group 4 received both T0901317 and in vivo LNA anti-miR-33. All treatments were administrated through intraperitoneal injection (IP). After 7 days and at the end of the study, mice were sacrificed, liver tissues were excised and blood samples were collected. LXR-α and ABCA1 genes and protein expression were quantified by real-time polymerase chain reaction (PCR) and western blotting, respectively.

RESULTS: LXR activation caused LXR-α and ABCA1 mRNA (P < 0.050) and protein elevation as compared to control (P < 0.001). miR-33 inhibition attenuates T0901317 effect on LXR-α expression in group IV. Co-administration of T0901317 and anti-miR-33 remarkably elevated high-density lipoprotein cholesterol (HDL-C) levels, compared to control group (P = 0.001). Separate administration of T0901317 and anti-miR-33 also elevated HDL-C levels (P < 0.010).

CONCLUSION: Co-administration of T0901317 and anti-miR-33 can be considered as a good therapeutic alternative for atherosclerosis because miR-33 inhibition reduced lipogenic effects of LXR-α activator and also helps LXR-α agonist to increase reverse cholesterol transport (RCT) and also HDL-C as antiatherogenic effects.

Keywords: Atherosclerosis, mir-33 Human, T0901317, Liver X Receptor-Alpha, ABCA1 Protein

Introduction

Dysregulation of lipid homeostasis is related to cardiometabolic and cardiovascular diseases.[1] Atherosclerosis is a progressive and multi-factorial disorder. Cholesterol-laden macrophages are the hallmark of atherosclerotic plaques.[2,3] Finding strategies in order to reduce the risk of cardiometabolic disease is the main goal of researchers. To achieve this goal, considerable attention has been paid to elevate the high-density lipoprotein cholesterol (HDL-C) levels and improve the reverse cholesterol transport (RCT).[4-6]

Cholesterol removal from tissues occurs via the liver. The process of cholesterol efflux from peripheral tissues and transport to the liver by HDL-C is known as RCT.[1,7] adenosine triphosphate-binding cassette transporter A1 (ABCA1) is necessary for cholesterol trafficking in RCT process. It also has a pivotal role in HDL-C formation.[1,4,8]

Liver X receptors (LXRs) are nuclear transcription factors which regulate lipid homeostasis. LXRs are comprised of two isoforms, designated as LXR-α and LXR-β, which are

1- Professor, Physiology Research Center AND Department of Clinical Biochemistry, Afzalipour School of Medicine, Kerman University of Medical Sciences, Kerman AND Sirjan School of Medical Sciences, Sirjan, Iran
2- Assistant Professor, Physiology Research Center AND Department of Clinical Biochemistry, Afzalipour School of Medicine, Kerman University of Medical Sciences, Kerman, Iran
3- Physiology Research Center AND Department of Clinical Biochemistry, Afzalipour School of Medicine Kerman University of Medical Sciences, Kerman, Iran
4- Professor, Physiology Research Center AND Department of Physiology, Afzalipour School of Medicine, Kerman University of Medical Sciences, Kerman, Iran
Correspondence to: Beydolah Shahouzehi, Email: bshahouzehi@yahoo.com

identified as members of orphan receptors superfamily. Recently there have been some reports showing cholesterol derivatives, such as oxysterols, act as physiological ligands of LXRs.[9,10] Synthetic LXR agonist, T0901317, is also capable to bind to and activating LXRs in the liver.[11] LXRs target genes are including sterol regulatory element-binding proteins (SREBPs), ABCA1, ABCG1, ABCG5, ABCG8, and cholesterol 7-α-hydroxylase (CYP7A).[10-13] LXRs can be encouraging therapeutic targets for cardiovascular disease prevention and treatment because there are beneficial effects following LXR activation on lipid homeostasis.[9,10,12] LXRs play a pivotal role in RCT regulation which occurs by direct LXR effects on ABCA1 and ABCG1.[13] LXR activation results in HDL-C elevation and reduced cholesterol levels.[14] Some studies have shown that LXR activation, by a synthetic ligand, increased expression of SREBP-1c and fatty acid synthase (FAS) and cause elevation of triglyceride (TG) levels.[9,10,13,15] Due to the lipogenic properties of synthetic agonists of LXR, their utilization as a therapeutic target in atherosclerosis would be in doubt.

microRNAs are endogenous, short (18-25 nucleotides) non-coding RNAs which bind to their target mRNA in order to inhibit its translation or degrade it.[1] Najafi-Shoushtari discovered that there are two microRNAs in the introns of SREBP-1 and SREBP-2 genes known as miR-33b and miR-33a, respectively.[1] miR-33a/b have very similar sequence and their seed sequence is exactly the same. Therefore, both have the same mRNA targets. miR-33 is involved in cholesterol and lipid metabolism and regulates ABCA1 and adenosine monophosphate-activated protein Kinase (AMPK). miR-33 inhibits ABCA1 expression and results in increased intracellular cholesterol levels.[1] Also, it has been demonstrated that miR-33 antisense oligonucleotides and genetic depletion of miR-33a in mice increased HDL levels.[1]

AMPK is an energy sensor which elevates fatty acid β-oxidation and also reduces synthetic pathways such as cholesterol and TG synthesis. AMPK activation attenuates LXR-induced steatosis in mice liver.[16] Moreover, AMPK activation reduces SREBP-1c mRNA expression which is increased by T0901317.[17,18] miR-33 elevation increases intracellular cholesterol levels and reduce RCT which results in generation and deterioration of atherosclerosis and plaques formation.[1,15] In this study, we evaluated the effects of miR-33 inhibition and LXR activation and the combination of both on LXR-α and ABCA1 expression in mice liver and circulating HDL levels.

Materials and Methods

T0901317, as LXR agonist, was obtained from Cayman Chemical (Ann Arbor, Michigan, USA, Cat no. 71810), in vivo locked nucleic acids (LNA)™ miR-33 inhibitor was purchased from Exiqon (Woburn, MA, United States). EZ-10 spin column total RNA mini-prep super kit (Bio Basic Inc., Markham, Canada), Primescript reverse transcriptase (RT) reagent kit (Takara Bio Inc., Japan), SYBR Premix Ex Taq II (Tli RNase H Plus) Takara Bio Inc., Japan), LumiFilm Chemiluminescent detection film (Roche, Germany), Phenylmethanesulfonyl Fluoride (Sigma, USA), sodium orthovanadate (Sigma, USA), protease inhibitor cocktail (Sigma, USA), and Amersham enhanced chemiluminescence (ECL) prime western blotting detection reagent (GE Healthcare UK Ltd.) were also used in this study. ABCA1 (sc-20794), β-Actin (sc-130656), LXRα/β (sc-13068), and goat anti-rabbit IgG-HRP (sc-2004) were purchased from Santa Cruz (Sana Cruz Biotechnology, USA). Real-time polymerase chain reaction (PCR) was performed on ABI StepOnePlus instrument (Applied Biosystem, Step One Plus, USA).

Mice were purchased from Animal Care Center of Kerman Physiology Research Center, Iran. Animals were kept for a week in an animal care facility for acclimatization. Then, mice weighing 22 ± 2 g were selected for the study and housed at a temperature of 22 °C, 12-hour light/12-hour darkness cycle. Animals had open access to standard chow diet and water. Selected mice were randomly allocated into four groups (n = 6) as follow: group 1, the control untreated group, received standard chow diet, group 2 received 30mg/kg/48 hour of T0901317, group 3 received 1mg/kg/48 hour of LNA miR-33 inhibitor, and group 4 received combination of LXR agonist and LNA miR-33 inhibitor as groups 2 and 3. Duration of the study was a week and animals in treatment groups received 3 intraperitoneal injections (IP) every two days. At the end of the study, after 10-hour fasting overnight, mice were sacrificed, blood samples were collected and serum was separated for HDL-C measurement. Also, liver tissues were excised and washed with cold saline and frozen in liquid nitrogen immediately and maintained at -80 °C for further examination. All procedures were approved by the Animal Research Ethics

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Table 1. Sequences of real-time polymerase chain reaction (PCR) primers

Gene	Forward primer 5' to 3'	Reverse primer 5' to 3'	Product size (kb)
ABCA1	GCTCTCAGGTGGGATGCAG	GGCTCGTCCAGAATGACAAC	81
β-Actin	CAACGAGCGGTTCCGATG	GCCACAGGATTCCATACCCA	90
LXR-α	AGGAGTGTCGACTTCGCAAA	CTCTTCTTGCCGCTTCAGTTT	101

ABCA1: Adenosine triphosphate-binding cassette transporter A1; LXR-α: Liver X receptor alpha

Committee of Kerman University of Medical Sciences, Iran (ethics committee permission number: IR.KMU.REC.1394.319).

Total RNA was extracted by EZ-10 spin column total RNA mini preps super kit according to company's instructions. For this purpose, 75 mg of liver tissue was excised from frozen storage and used for RNA extraction. For cDNA synthesis, we used 250 ng of total RNA and PrimeScript RT reagent kit, using Oligo-dT and random 6mer primers. Real-time PCR was performed by SYBR Premix Ex Taq II (Tli RNase H Plus). The reactions contained ROX (6-Carboxyl-X-Rhodamine), forward and reverse primers, sterile water, and 100 ng cDNA. Real-time PCR was performed on ABI StepOnePlus instrument. The thermal reaction was stage 1 denaturation, 95 °C for 10 minutes, stage 2, 40 cycles of 95 °C for 20 seconds and 60 °C for 30 seconds. Furthermore, the melt curve analysis was performed which started from 60 °C and increased by 0.3 °C. Primers (Table 1) were purchased from Macrogen (Macrogen Inc., Seoul, South Korea). Finally, the expression level was determined by the $2^{-\Delta\Delta Ct}$ method and normalized to β-actin as the housekeeping gene.[19,20]

For tissue homogenization and protein extraction, we used RIPA buffer (containing protease inhibitor cocktail, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM sodium orthovanadate, pH 7.4). About 50 mg of liver tissue was excised and homogenized in ice-cold RIPA buffer by sonication (Hielscher Ultrasound, UP200H, Germany). Homogenates were centrifuged at 15000 rpm, 4 °C for 20 minutes and supernatant was removed for further study. Total protein concentration in the supernatant was measured by the Bradford method. In order to run sodium dodecyl (lauryl) sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), an equal volume of 2× sample buffer was added to each sample and incubated at 95 °C for 5 minutes. Then, 100 µg of total protein was loaded into each well of a 12.5% gel and separation was conducted by a constant voltage (120 V, 80 min, Tris-Gly buffer, pH 8.3). After electrophoresis, proteins were transferred to a Polyvinylidene Difluoride (PVDF) membrane at a constant current 220 mA in cold transfer buffer.

The membrane was incubated with blocking buffer (5% skim milk in Tris-buffered saline and Tween 20) overnight at 4 °C. In the next step, the membrane was washed 4 times, 5 minutes each. Washed membrane was incubated with primary antibody for 1 hour, followed by a washing step for 4 times. Eventually, incubation with secondary antibody for 1 hour was carried out, followed by 4 times washing, and the PVDF membrane was incubated with Western Lightning Plus ECL for 1 minute. The antigen-antibody complex was detected by enhanced chemiluminescence detection film in a dark room. Band densities were analyzed by the ImageJ software. β-Actin was used as the housekeeping gene and control.[21]

Real-time PCR and western blotting data, and HDL-C levels were reported as a mean ± standard error of the mean (SEM). Data were analyzed by SPSS software (version 19, SPSS Inc., Chicago, IL, USA). Comparison between groups was conducted by analysis of variance (ANOVA) and pair-wise comparison was done by post hoc Tukey's test. $P \leq 0.050$ was considered as statistically significant.

Results

We showed that anti-miR-33 reduced miR-33 levels by real-time PCR (data are not shown here). LXR activation by LXR synthetic agonist T0901317 elevated LXR-α gene expression compared to control ($P = 0.046$) (Figure 1A). In contrast, anti-miR-33 reduced LXR-α gene expression which was attenuated by a combination of both T0901317 and anti-miR-33. ABCA1 expression was elevated by T0901317 ($P = 0.033$), anti-mR-33 ($P < 0.001$) and the combination of both, but only when they were administrated separately the elevation was significant compared to control group. In fact, a combination of T0901317 and anti-miR-33 insignificantly increased ABCA1 gene expression (Figure 1B).

LXR-α protein levels were significantly increased by T0901317 administration compared to control ($P < 0.001$). On the other hand, anti-miR-33 and combination of anti-miR-33 plus T0901317 significantly reduced LXR-α protein levels compared to T0901317 ($P < 0.001$) (Figure 2A).
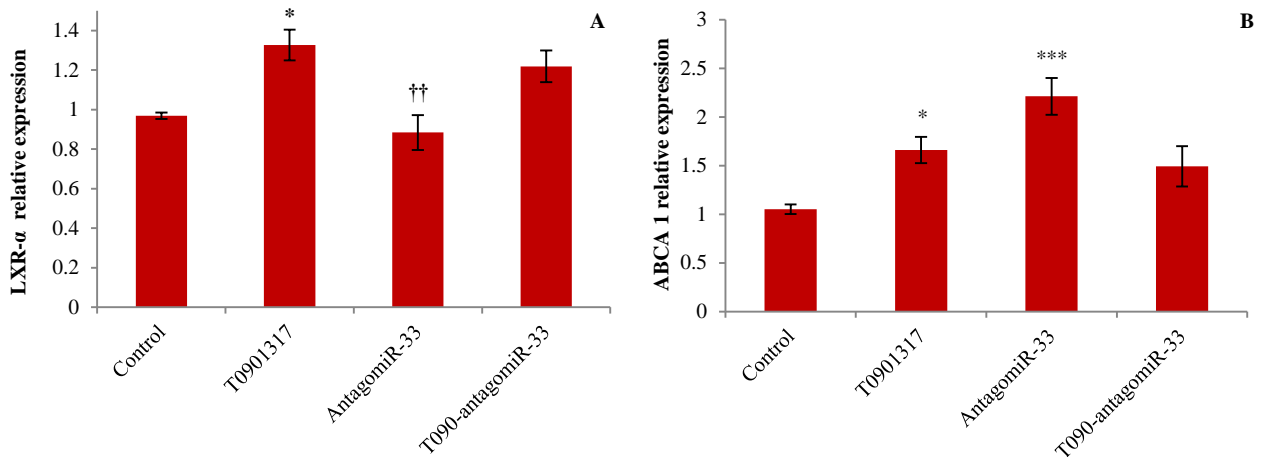
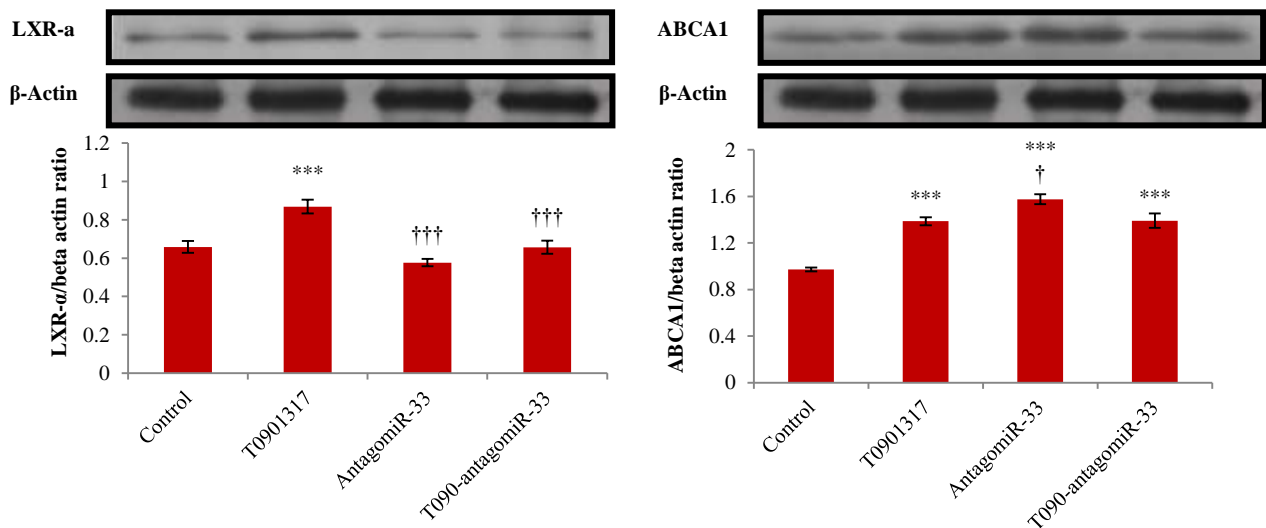**Figure 1.** Expression of liver X receptor (LXR)-α (A) and adenosine triphosphate-binding cassette transporter A1 (ABCA1) (B) genes was evaluated by real-time polymerase chain reaction (PCR). Control group received standard mice chow diet, T0901317 group received T0901317 by intraperitoneal injection (IP), AntagomiR-33 group received anti-miR-33 by IP, and group T090-antagomiR-33 received combination of both. Data are expressed as mean ± standard error of the mean (SEM). * statistically significant compared to the control group; † statistically significant compared to the T0901317 group (* P < 0.050, *** P < 0.001, †† P < 0.010)

ABCA1 protein expression was elevated in other three groups compared to control group (P < 0.001), also, anti-miR-33 therapy increased ABCA1 protein levels significantly compared to T0901317 group (P < 0.050) (Figure 2B). HDL-C increased in three other groups compared to control, T0901317 and antimiR-33 elevated HDL-C significantly (P < 0.010). However, the group that received antimiR-33 plus T0901317 caused more significant elevation of HDL-C (P = 0.001) (Figure 3).

Discussion

LXR activation by T0901317 elevated LXR-α expression and we found that anti-miR-33 administration attenuates T0901317 increasing effect on LXR-α expression. By increasing ABCA1 and ABCG1, LXR-α increases HDL-C which is an antiatherogenic factor.[2]

Figure 2. Protein expression of liver X receptor (LXR)-α (A) and adenosine triphosphate-binding cassette transporter A1 (ABCA1) (B) was evaluated by western blotting. Control group received standard mice chow diet, T0901317 group received T0901317 by intraperitoneal injection (IP), AntagomiR-33 group received anti-miR-33 by IP, and groupT090-antagomiR-33 received a combination of both. Data are expressed as mean ± standard error of the mean (SEM). * Statistically significant compared to the control group, † Statistically significant compared to the T0901317 group (*** P < 0.001, ††† P < 0.050)

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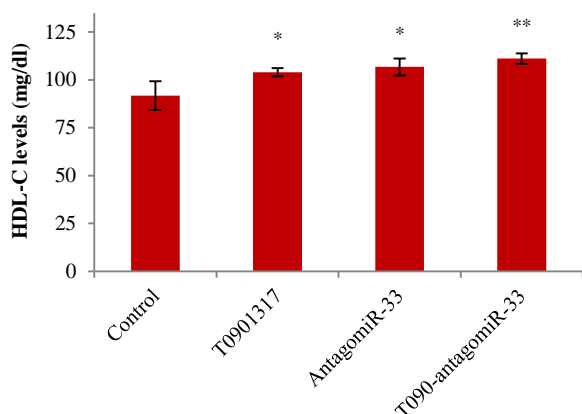
Figure 3. High-density lipoprotein cholesterol (HDL-C) levels were elevated by T0901317, anti-miR-33 and co-administration of T0901317-antagomiR-33. Control group received standard mice chow diet, T0901317 group received T0901317 by intraperitoneal injection (IP), AntagomiR-33 group received anti-miR-33 by IP, and groupT090-antagomiR-33 received combination of both. Data are expressed as mean ± standard error of the mean (SEM).
* statistically significant compared to the control group
(* P < 0.010, ** P < 0.001)

The beneficial and therapeutic effects of LXR-α induction over atherosclerosis would be limited because of promotion of lipogenesis which may result in hepatic steatosis or possibility of exacerbating insulin resistance-related disease.[10,22] We showed that LXR activation and miR-33 inhibition have different effects on LXR-α expression. Thus, T0901317 increased and anti-miR-33 reduced LXR-α expression, while anti-miR-33 therapy along with T0901317 reduced LXR-α expression. Consistent with our results, Horie et al. showed that miR-33 deficiency elevated cholesterol efflux from macrophages and promote HDL-C production resulting in regression of atherosclerosis.[5] Finally, our findings showed that anti-miR-33 therapy helps T0901317 to increase HDL-C levels and also, miR-33 inhibition reduced LXR-α lipogenic effects. It has been showed that anti-miR-33 therapy increases AMPK and ABCA1 expression.[1] AMPK has negative effects on synthetic pathways through impeding cholesterol and TG synthesis by inhibition of LXR-α, SREBPs and 3-Hydroxy-3-Methylglutaryl-CoA Reductase (HMGCR).[23] We conclude that anti-miR-33 administration, which is well-documented AMPK up-regulator, inhibits LXR-α/SREBP-1c lipogenesis pathway probably by an AMPK dependent mechanism.

ABCA1 helps cholesterol trafficking. It is also a major component of RCT and HDL-C production.[4,24] We found that separate administration of either T0901317 or anti-miR-33 increased ABCA1 expression. Co-administration of T0901317 and anti-miR-33 attenuated ABCA1 gene expression but still, ABCA1 expression is more than the control group. Previous studies have demonstrated that ABCA1 reduced intracellular cholesterol content which stimulates SREBP-2 expression.[6-8] SREBP-2 is a transcription factor increasing HMGCR expression and elevates cholesterol biosynthesis.[6] According to the recent reports, it has been proved that miR-33 is coexpressed along with SREBP-2 gene.[25,26] miR-33 inhibits ABCA1 mRNA and reduces ABCA1 levels.[27] Therefore, we conclude that this reduced ABCA1 expression by combination of T0901317 plus anti-miR-33, compared to either T0901317 or anti-miR-33, probably is a consequence of miR-33 slight elevation (miR-33 data are not shown) in this group. However, there was a significant elevation of ABCA1 protein expression in all three groups compared to the control.

miR-33 regulates intracellular cholesterol levels. Overexpression of miR-33 declines cholesterol efflux and in contrary, miR-33 inhibition causes elevation of cholesterol efflux by LXR activation.[9,27] LXR activation in peripheral tissues such as macrophages increases RCT and cholesterol transport to the liver. In liver, LXR synthetic agonists lead to cholesterol excretion.[7,10] Inhibition of miR-33 activity by anti-miR-33 in peripheral tissues helps LXR activation to increase ABCA1 levels and may improve RCT as a synergic effect.[28] Li et al. demonstrated that AMPK activation reduced hepatic steatosis by inhibition of SREBPs activity. They introduced this mechanism as a new therapeutic strategy for atherosclerosis and dyslipidemia.[29] We showed that miR-33 inhibition along with T0901317 administration improved ABCA1 protein levels significantly; on the other hand, anti-miR-33 therapy reduces LXR-α expression. Anti-miR-33 increases AMPK levels which inhibit LXR-α and SREBP-1c levels, so it seems that miR-33 suppression by anti-miR-33 administration in liver attenuates LXR agonist lipogenic effects and can help to reduce steatohepatitis.

We showed that LXR activation or miR-33 inhibition lead to HDL-C elevation (P < 0.050). Moreover, co-administration of anti-miR-33 and T0901317 remarkably increased HDL-C levels (P < 0.001). Yamamoto et al. showed that hepatic

ABCA1 inhibition reduced plasma HDL-C but increased RCT and cholesterol excretion as bile.[7] We also found that co-administration of T0901317 and anti-miR-33 reduced ABCA1 gene expression which is consistent with Yamamoto et al. findings. However, we did not observe this effect on ABCA1 protein levels.[7] Yamamoto et al. reported that HDL-C was reduced after ABCA1 decline.[7] On the contrary, we observed a significant elevation of HDL-C levels after co-administration of T0901317 and anti-miR-33 and slight reduction of hepatic ABCA1 expression. According to previous studies, this HDL-C increase could probably result from LXR activation and miR-33 inhibition in peripheral tissues, which return cholesterol by ABCG1 and ABCA1 to circulation.[10,28]

Conclusion

miR-33 inhibition is a potential therapeutic approach which elevates HDL-C levels. Thus, it may be a potential therapeutic target to reduce the risk of cardiovascular disease. Also, many studies have shown that LXR activation has beneficial effects on lipid metabolism and atherosclerosis. They have also suggested that this could be a novel target for cardiovascular disease treatment. Our results showed that combination of T0901317 and miR-33 suppression remarkably elevate HDL-C production and also reduce LXR-α expression, the latter being totally dependent on miR-33 inhibition. Furthermore, they also reduced LXR-α expression which is a master regulator of TG synthesizing genes. Therefore, administration of a combination of miR-33 inhibition and LXR activation can be considered as a therapeutic approach in cardiovascular and cardiometabolic disorders.

Acknowledgments

Conflict of Interests

Authors have no conflict of interests.

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