LXR Agonist T0901317-Induced Hyperlipidemia Does Not Lead to Lipid Accumulation in the Rat Heart

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Key Words
Liver X receptors • Cardiac muscle • Fatty acids • Diacylglycerol • Cholesterol

Abstract
Background/Aims: Liver X receptors (LXRα and LXRβ) are ligand-activated transcription factors that regulate expression of genes involved in lipid and cholesterol metabolism. LXR expression has been identified in human and rodent cardiac tissue, however, its role in this tissue remains unclear. The aim of this study was to investigate effects of in vivo LXR activation on lipid metabolism in the rat myocardium under the conditions of low and high lipid intake.
Methods: The experiments were performed on male Wistar rats fed for 5 weeks on either low fat diet (LFD) or high fat diet (HFD). Next, the animals were randomly divided into two groups receiving either LXR agonist – T0901317 (10mg/kg/d) or vehicle for the last week of the experiment. After anesthesia samples of the left ventricle and blood were taken.
Results: It was found that LXRβ is the dominant isoform in the rat myocardium and the expression of both LXR isoforms did not change after administration of T0901317. Agonist treatment induced hyperlipidemia in low fat fed rats and this effect was amplified in high fat fed rats. LXR agonist elevated content of myocardial triacylglycerols in animals fed on LFD and content of phospholipids in animals fed on HFD. Levels of the remaining examined lipid classes (nonesterified fatty acids, diacylglycerol, free cholesterol, cholesterol esters, ceramide) was decreased or unchanged after LXR activation. Conclusion: We conclude that administration of T0901317 does not lead to severe myocardial lipid accumulation in rats despite of its high plasma availability.

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Introduction

Liver X receptors (LXRs) are ligand-activated transcription factors that belong to the nuclear receptor superfamily [1]. There are two LXR isoforms termed LXRα and LXRβ. LXRβ is expressed in all examined tissues, whereas LXRα expression is restricted to tissues with high level of lipid metabolism, such as liver, adipose tissue, intestine, macrophages and lungs [2]. Upon activation both isoforms form heterodimers with retinoid X receptor (RXR), and subsequently the LXR/RXR complex binds to the liver X receptor response element in the promoter region of the target genes and their transcription is stimulated [3]. Endogenous LXR ligands are oxysterols, oxygenated derivatives of cholesterol [4]. Synthetic LXR agonists T0901317 and GW3965, are commonly used in experimental studies and they activate both LXR isoforms with similar potency [3].

LXRβ expression has been identified in human and rodent cardiac tissue [2, 5-7]. LXRβ is the dominant isoform in the heart of mice [2, 5, 8]. Stimulation of LXRs with T0901317 in mice causes an increase in cardiac expression of genes involved in lipid and cholesterol metabolism, such as sterol response element binding protein 1c (SREBP-1c), stearoyl-CoA desaturase 1 (SCD1), ATP-binding cassette transporters A1 and G1 (ABCA1, ABCG1), known LXR target genes [9, 10]. In addition the LXR agonist GW3965 increased intracellular triglyceride level in murine heart and isolated cardiomyocytes [8].

The most important role of LXRs is maintaining of cholesterol homeostasis. When the amount of cholesterol increases there is a subsequent accumulation of intracellular oxysterols [11]. It causes LXR activation which induces the transcription of genes involved in reverse cholesterol transport [3], its conversion to bile acids in the liver [12], excretion of cholesterol directly into bile [13] and decreasing intestinal cholesterol absorption [3, 11, 12, 14]. In addition, synthetic LXR agonists stimulate hepatic free fatty acid uptake and hepatic lipogenesis by induction of expression of fatty acid translocase/CD36 (FAT/CD36) [15] and SREBP-1c, respectively, which results in liver steatosis and hypertriglyceridemia [16, 17]. Administration of LXR agonist, T0901317 or GW3965, normalizes also plasma glucose level and enhances insulin sensitivity in rodent models of type 2 diabetes and insulin resistance [18, 19].

Cardiac muscle is one of the most metabolically active tissues in the body and must continually generate ATP at a high rate for its contractile activity. Fatty acids are the main source of energy for the heart in the normal state [20]. The rate of fatty acid uptake depends on the concentration of fatty acids in the plasma [21]. Hyperlipidemia may cause lipid accumulation in the heart resulting from an imbalance between nonesterified fatty acids (NEFA) uptake and utilization [22].

LXRs as transcription factors regulate lipid metabolism in various tissues and cells. LXR expression has been noted in cardiac tissue, however its role in myocardial lipid homeostasis remains unclear. Lipids play many important roles in cardiomyocytes, for example they are energetic substrates, they determine fluidity and permeability of membranes, moreover they are precursors of signaling molecules and ligands for transcription factors. Therefore, the aim of this study was to investigate effects of in vivo LXR activation by the synthetic agonist T0901317 on lipid metabolism in the rat myocardium under the conditions of low and high lipid intake.

Materials and Methods

Animals

The investigation was approved by the Ethical Committee for Animal Experiments at the Medical University of Bialystok (permit no. 35/2008). Male Wistar rats (80-100g) were housed under controlled conditions (21°C ± 1, 12 h light/12 h dark cycle, light on at 06:00 am) with unlimited access to water and laboratory rat chow. The animals were randomly assigned to either low-fat diet (LFD) or high-fat diet
(HFD). Low fat diet (LSM, Agropol, Motycz, Poland) contained 16% of metabolizable energy from fat, 62% from carbohydrates, and 22% from proteins. High-fat diet (D12492 Research Diets, Inc.) contained 60% of metabolizable energy from fat, 20% from carbohydrates, and 20% from proteins. Next, the animals were randomly divided into two groups receiving either vehicle or a dual LXRα/β agonist T0901317 (Cayman Chemicals, 10 mg/kg/d suspended in 0.5% carboxymethylcellulose) for the last week of the experiment. The solutions were administrated once daily in the morning by an oral gavage. After five weeks of diet, the 24h after the T0901317/vehicle administration, all rats were anaesthetized by intraperitoneal injection of pentobarbital in the dose of 80 mg/kg (in the fed state). Blood taken from the abdominal aorta was collected in heparinized tubes, centrifuged, the plasma separated and flash-frozen in liquid nitrogen. Next samples of the left ventricle were excised and immediately freeze-clamped with aluminum tongs precooled in liquid nitrogen. All samples were stored at -80°C until analysis.

Myocardial lipids

Ventricular samples were pulverized in an aluminum mortar precooled in liquid nitrogen. Lipids were extracted by the method of Folch. The fractions of total phospholipids, triacylglycerols, diacylglycerols, NEFA, free cholesterol and choleseter esters were separated by thin-layer chromatography according to Roemen and van der Vusse [23]. Lipid were then transmethylated in either 1 M methanolic sodium methoxide (Fluka) at room temperature for 10 minutes (triaclyglycerols and phsopholipids) or 14% methanolic boron trifluoride (Sigma) at 100°C for either 2 (NEFA) or 10 (diacylglycerols) minutes. The content of resulting fatty acid methyl esters was determined using gas-liquid chromatography as previously described [24]. Free cholesterol and cholesterol esters were eluted from the gel with chloroform, evaporated under nitrogen stream and redissolved in 2-propanol or diethyl ether, respectively. The content of free cholesterol and cholesterol esters was subsequently measured with commercially available cholesterol diagnostic kit (BioMaxima). The content of ceramide was determined as previously described [25]. Briefly, tissue lipids were extracted into chloroform and the samples were then subjected to alkaline hydrolysis to deacylate ceramide. Free sphingosine liberated from ceramide was converted to o-phthalaldehyde derivative and analyzed using HPLC system. N-palmitoyl-D-erythrosphingosine (C17 base) was used as an internal standard.

Real-time PCR

Total RNA was isolated from 50mg of frozen tissue using Total RNA (A&A Biotechnology) according to the producer's instructions. First strand cDNA was generated using High Capacity cDNA Reverse Transkription Kit (Applied Biosys) according to the producer's instructions. PCR products were obtained by amplification of cDNA using primers as follows: forward: 5’-TATGCTGTTATTTGCTGCTCC-3’; reverse: 5’-GGTCTACACGCTTCATAGG-3’ for LXRα; forward: 5’-CTCTTTGTCACACCCCTC-3’; reverse: 5’-TCCTGTCGCTCTGCTC-3’ for LXRβ; forward: 5’-CACACAAATGCAATCCGTTT-3’; reverse: 5’-GGCCTTGACCTTGTTCATGT-3’ for PPARα; forward: 5’-AACATCCCGACCTACACGCTTGG-3’; reverse: 5’-CTCTGGCAAGAACATCTTGTTGG-3’ for PPARδ; forward: 5’-CAAGCCCGCCACCAGTTGC-3’; reverse 5’-GTAGGAGTCTTCTGACCACATAC-3’ for β-actin.

PCR was performed with SYBR Green JumpStart Taq ReadyMix (Sigma), using a Bio-Rad Chromo4 system. Reaction mix in final volume 25µl contained 12,5µl SYBR Green I, 3µl cDNA and 300nM of each primer pairs. PCR was carried out under following conditions: 15 sec denaturation at 54°C, 1 min annealing at 55°C for PPARα, 57°C for LXRα, 58°C for LXRβ, 63°C for β-actin, 1 min extension at 72°C for 40 cycles. PCR efficiency was examined by serial diluting the template cDNA, and a melt curve was performed after each reaction to verify PCR product specificity. A sample containing no cDNA was used as a negative control to verify the absence of primer dimmers. The results were normalized to β-actin expression measured in each sample. Relative expression of genes was calculated according to Pfaffl metod [26].

Western blotting

Myocardial samples were lysed in the RIPA buffer (pH 8.0 50 mM Tris–HCl, 150 mM NaCl, 1 % NP-40, 0.5 % sodium deoxycholate1 mM EDTA, protease and phosphatase inhibitors). Protein concentrations were measured using Bichinchoninic Acid Solution (Sigma). Proteins were separated using 10% SDS-polyacrylamide gel electrophoresis. Next, proteins were transferred to nitrocellulose membranes and the membrane was probed with primary antibodies against LXRα, LXRβ, FAT/CD36, β-actin (Abcam), SREBP-1
Plasma measurements

Concentration of plasma glucose, triacylglycerols, NEFA and total cholesterol were determined with the use of Glucose Oxidase Reagent Set (Pointe Scientific Inc.), Serum Triacylglycerol Determination Kit (Sigma), wako NEFA C Kit (Wako Chemicals) and Cholesterol Kit (BioMaxima), respectively.

Statistical analysis

Data are presented as means ± SD. Statistical analysis was performed using Statistica 9 (StatSoft). Statistical comparisons between experimental groups were made by using two-way ANOVA followed by Newman-Keuls post hoc test. P values <0,05 were considered statistically significant.

Results

Administration of T0901317 did not affect body weight or food consumption either in animals fed on LFD or in animals fed on HFD (Table 1). LXR activation resulted in a decrease in plasma glucose level in animals fed on either diet (Table 1). Plasma cholesterol, NEFA and triacylglycerols concentrations were increased after HFD feeding, however in the case of triacylglycerols the difference was not statistically significant (Table 1). LXR activation resulted in a further increase in plasma level of NEFA and triacylglycerols and the same changes were observed in the case of animals fed on LFD (Table 1). T0901317 did not affect plasma cholesterol level in animals fed on either LFD or HFD diet (Table 1).

There were no significant changes in the myocardial content of diacylglycerols and free cholesterol regardless of a diet and administration of T0901317 (Fig. 1). Administration of LXR agonist to rats fed on LFD decreased the content of myocardial ceramide and cholesterol esters, whereas the content of triacylglycerols was elevated (Fig. 1). High-fat diet caused accumulation of free fatty acids, triacylglycerols and phospholipids but reduced the level of ceramide in the heart. Activation of LXR receptors in rats fed on HFD elevated myocardial content of phospholipids, but decreased the content of myocardial NEFA and ceramide and did not affect the level of the remaining examined lipid classes (Fig. 1).

Both isoform of LXR were expressed in the rat heart as revealed by real-time PCR (Fig. 2) and LXRβ was more abundant isoform than LXRα (estimated difference after correction for PCR reaction efficiency was 2,7-fold, data not shown). T0901317 administration did not change myocardial mRNA expression of LXR isoforms either in animals fed on low fat diet or in animals fed on high fat diet (Fig. 2). LXR agonist did not induce changes in expression of

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**Table 1.** Effect of T0901317 treatment on body weight, food intake and plasma measurements in a fed state. The results are means ± SD (n=10). * - significant difference versus low fat diet (LFD) vehicle group. # - significant difference versus high fat diet (HFD) vehicle group. NEFA – nonesterified fatty acids

|                  | LFD         | T0901317   | HFD         | T0901317   |
|------------------|-------------|------------|-------------|------------|
| Initial body weight (g) | 93.46 ± 7.90 | 94.40 ± 7.49 | 91.43 ± 9.61 | 94.27 ± 8.98 |
| Final body weight (g) | 338.08 ± 29.75 | 344.10 ± 27.66 | 383.36 ± 58.94 * | 365.53 ± 33.41 |
| Caloric intake (kcal/d/rat) | 104.96 ± 10.16 | 107.28 ± 13.81 | 117.54 ± 11.41 | 100.23 ± 14.87 |
| Glucose (mg/dl) | 156 ± 17 | 133 ± 11 * | 167 ± 15 | 147 ± 22 # |
| NEFA (nmol/ml) | 183 ± 32 | 246 ± 44 * | 361 ± 100 * | 631 ± 220 # |
| Triacylglycerols (nmol/ml) | 863 ± 64 | 1985 ± 740 * | 1907 ± 980 # | 6094 ± 3113 # |
| Total cholesterol (nmol/ml) | 1650 ± 363 | 1268 ± 336 | 2390 ± 661 * | 2619 ± 546 |
Harasiuk et al.: T0901317 Does Not Lead to Lipid Accumulation in the Heart

**Fig. 1.** Effect of T0901317 treatment on myocardial content of examined lipids. The results are means ± SD (n=10). * - significant difference versus low fat diet (LFD) vehicle group. # - significant difference versus high fat diet (HFD) vehicle group. NEFA – nonesterified fatty acids.

PPARα and PPARδ genes in animals on low fat diet (Fig. 2). High fat diet increased the level of PPARs mRNA in the rat heart (Fig. 2). T0901317 administration in high fat diet group decreased mRNA expression of PPARα and PPARδ compared to group with high fat diet alone.
Harasiuk et al.: T0901317 Does Not Lead to Lipid Accumulation in the Heart

Table 2 lists average cycle threshold (Ct) values of measured genes determined after real-time PCR. The results are means ± SD (n=6). Ct – threshold cycle. LXRα – liver X receptor α. LXRβ – liver X receptor β. PPARα – peroxisome proliferator-activated receptor α. PPARδ – peroxisome proliferator-activated receptor δ.

Table 2. Average Ct values of measured genes determined after real-time PCR. The results are means ± SD (n=6). Ct – threshold cycle. LXRα – liver X receptor α. LXRβ – liver X receptor β. PPARα – peroxisome proliferator-activated receptor α. PPARδ – peroxisome proliferator-activated receptor δ.

| Genes | LFD Vehicle | T0901317 | HFD Vehicle | T0901317 |
|-------|-------------|-----------|-------------|-----------|
| LXRα  | 24.60 ± 0.37| 25.98 ± 0.52| 25.11 ± 0.47| 23.24 ± 0.67|
| LXRβ  | 21.87 ± 0.35| 23.43 ± 0.76| 22.50 ± 0.47| 20.75 ± 0.84|
| PPARα | 20.45 ± 0.38| 21.23 ± 0.59| 19.92 ± 0.61| 19.87 ± 0.75|
| PPARδ | 20.33 ± 0.38| 21.15 ± 0.69| 20.20 ± 0.23| 19.49 ± 0.51|

Fig. 2. Effect of T0901317 treatment on mRNA expression of selected genes in the heart. Fold change vs. low fat diet (LFD) vehicle group. The results are means ± SD (n=6). * - significant difference versus LFD vehicle group. # - significant difference versus high fat diet (HFD) vehicle group. LXRα – liver X receptor α. LXRβ – liver X receptor β. PPARα – peroxisome proliferator-activated receptor α. PPARδ – peroxisome proliferator-activated receptor δ.

Protein content of LXRα, LXRβ and FAT/CD36 in cardiac muscle of rat fed on low fat diet remained stable after treatment with T0901317 (Fig. 3). The agonist also had no impact on the level of these proteins in animals fed on high fat diet (Fig. 3). On the other hand, content of myocardial mature form of SREBP-1 decreased after LXR activation in both diet groups (Fig. 3).

Discussion

In the present study we found that both isoforms of LXR are expressed in the rat heart and that LXRβ is more abundant than LXRα. Our data obtained previously indicate also that
in rat skeletal muscles LXRβ expression is higher than LXRα [27]. Others reported that LXRβ is the dominant isoform in the heart and skeletal muscle of mice [2, 8, 28] and in human skeletal muscle [29, 30].

We did not observe changes in myocardial LXRs expression after administration of T0901317 either at mRNA or protein level. Similar observation for murine heart was reported by Lei et al. [8] where mRNA expression of both LXRs isoforms was unchanged after GW3965 injections. Treatment with T0901317 did not affect the expression levels of LXRα and LXRβ also in the mouse myotubes [28, 31]. It has been shown that LXRα expression is controlled by an autoregulatory mechanism and synthetic as well as natural LXR agonists increased LXRα expression in human macrophages, adipocytes, hepatocytes, skin fibroblasts and myotubes [30, 32-36]. Ulven et al. [37] observed that T0901317 increased the amount of LXRα mRNA in mouse white adipose tissue but not in other examined cells. Lack of changes of LXRs expression after administration of T0901317 in our study does not mean lack of activation of LXRs which were present in cardiomyocytes. Previous publications of other authors indicated activity of LXRs as transcription factors in the myocardium. Steffensen et al. [9] analysed LXR agonist-induced changes by genome-wide expression-profiling analysis of seven organs, including heart, from mice treated orally with T0901317. LXR agonist treatment altered expression profiles of genes involved in lipid, cholesterol and carbohydrate metabolism not only in liver, but also in adrenal, kidney, lung and heart [9]. Kuipers et al. [10] showed that like in other tissues, mRNA expression of specific target genes of LXR is increased in the heart of mice treated with T0901317, which means that LXR specific pathways are activated by T0901317 treatment in the myocardium.

We observed an increase in plasma triacylglycerols and NEFA concentration after T0901317 administration. This is well-known effect of LXRs activation that causes hyperlipidemia in rodents by stimulation of expression of hepatic lipogenic genes. As a result, the level of triacylglycerols, phospholipids and free fatty acids rises in plasma [16, 38, 39].
We did not observed changes in plasma cholesterol level and myocardial cholesterol content in animals fed on either LFD or HFD diet after treatment with T0901317. The content of myocardial cholesterol esters after T0901317 administration decreased in animals fed on LFD and did not change in animals fed on HFD. Reboulleau et al. [40] observed that treatment with a cocktail of LXR/RXR agonists (22-hydroxy-cholesterol/9-cis-retinoic acid) induced cholesterol efflux from neonatal rat cardiomyocytes and this is one of the mechanisms by which cells maintain their cholesterol pools. We may speculate that similar mechanism was activated in our in vivo settings. Cholesterol is a key component of cell membranes and maintenance of a constant cholesterol level in cardiomyocytes is required for proper functioning of the membrane-bound enzymes and cation transporters [41].

It can be expected that high plasma lipid concentration would lead to increased flux of free fatty acids into the myocardium, contributing to intracellular lipid accumulation. However we did not observe lipid accumulation in the rat heart after treatment with T0901317. Out of all examined lipid fractions only triacylglycerols content was moderately increased in the heart of animals fed on LFD after LXR activation. Sugden and Holness [42] reported cardiac triacylglycerol accumulation in mouse fed for 5 days with standard diet supplemented with T0901317. Similarly, Lei et al. [8] found increased level of triacylglycerols in mouse cardiac tissue after in vivo GW3965 injections. Furthermore GW3965 influenced triacylglyceride composition in favor of monounsaturates [8]. We also observed that T0901317 affected cardiac fatty acids profile of triacylglycerols in the rat heart, and this was reflected by increased content of monounsaturated fatty acids (data not shown). According to some researchers [30, 43, 44] accumulation of triacylglycerols in non-adipose tissues is not harmful, it is rather accumulation of intermediary metabolites, such as long-chain acyl-CoAs, ceramides and diacylglycerols that may have toxic effects on cells. Probably these intermediary metabolites did not accumulate in the cells during triglyceride synthesis [30, 45]. This is consistent with our observations, as triacylglycerols content was elevated, while ceramide content decreased after T0901317 treatment.

In animals fed on HFD a significant increase in plasma triacylglycerols and NEFA concentrations occurred after T0901317 administration. Despite increased availability of lipids in plasma we did not find myocardial lipid accumulation. LXR agonist slightly raised only phospholipid content. There was a decrease in NEFA and ceramide level and no change in concentration of other examined lipid fractions in the rat heart.

Why LXR-induced hyperlipidemia does not lead to myocardial lipid accumulation in rats? Firstly, LXRβ is the dominant isoform in the rat heart and perhaps activation primarily of this isoform caused observed changes. There is a tissue-specific expression of both isoforms of LXRs, so probably there are tissue-selective functions for them [2]. Moreover even the same isoform could create distinct changes because of the presence of specific intracellular coactivators, corepressors and endogenous ligands within different cells [9, 46-48].

Secondly, there were no changes in expression of myocardial FAT/CD36 protein after LXR activation in high fat diet fed animals. In heart as well as in skeletal muscle increase of the expression of FAT/CD36 is followed by the increase of fatty acid uptake [49].

Another possible explanation of the absence of lipid accumulation in the heart was the increase of the rate of myocardial lipids utilization after LXR activation. In our study we did not observe increase of expression of peroxisome proliferator-activated receptor α gene (PPARα) in the rat heart after administration of T0901317, however elevation of the efficiency of β-oxidation cannot be excluded. Wright et al. [50] reported increase of myocardial fatty acid utilization in mice after 5 weeks of HFD, although there were no change in mRNA level of PPARα. PPARα is a transcription factor and increased utilization of fatty acids might result in an increase in PPARα activity and not necessarily PPARα expression itself [51]. Sugden and Holness [42] demonstrated that T0901317 activation in vivo causes a marked enhancement of protein expression of cardiac pyruvate dehydrogenase kinase 4 (PDK4), enzyme which promotes fatty acid oxidation and is one of PPARα target genes [50]. Expression of PDK4 gene is not covered by this study, but we cannot rule out possibility that T0901317 have an impact on content and activity of myocardial enzymes involved in cardiac lipid metabolism.
Absence of lipid accumulation in the rat heart might be explained by inhibition of lipogenesis in cardiac cells. In the heart of animals treated with LXR agonist there was a decrease in the mature form of SREBP-1 protein level. SREBP-1c is a transcription factor that controls lipogenesis and is activated during overnutrition [52]. Marfella et al. [52] observed an increase in cardiac level of SREBP-1c and intramyocyte lipid accumulation in the heart of patients with metabolic syndrome [52]. According to Marfella et al. [52] these results imply that SREBP-1c could play a role in the regulation of intracellular lipid stores in the human heart, as it has been observed in the liver cells [53].

Cells of non-adipose tissues have a limited capacity for storage of lipids. When this capacity is exceeded, content of lipids may be reduced by its export from cells to plasma in the form of lipoproteins [22]. It appears that the heart, like liver or intestine, is a lipoprotein-secreting organ and is able to produce and secrete apolipoproteins B (apoB) [54-56]. ApoB secretion prevented cardiac triacylglycerols accumulation in fat-fed transgenic mice with overexpression of human apoB in the heart [57, 58]. In mice T0901317 stimulate, both in vivo and in vitro, the expression of apolipoprotein E in skeletal muscles [28], apolipoprotein D in adipocytes [59], apolipoprotein A-IV in hepatocytes [60]. In hamsters T0901317 led to increased plasma triglyceride apoB, due to enhanced hepatic secretion of apoB-containing lipoproteins [61]. It might be that LXR activation in rats causes increase of expression or secretion of apolipoproteins from the heart as a protection from lipid accumulation.

In summary we have found that LXRβ is the dominant isoform in the heart of rats. Administration of T0901317 caused increase of triacylglycerols and NEFA concentration in plasma, which is consistent with previous reports of other authors, and this applies to both diet group. LXR agonist elevated content of myocardial triacylglycerols in animals fed on LFD and content of phospholipids in animals fed on HFD, whereas the level of the remaining examined lipid classes was decreased or not affected after LXR activation. In conclusion administration of T0901317 induces hyperlipidemia but does not lead to severe lipid accumulation in the rat heart.

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Disclosure Statement

None declared

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Harasiuk et al.: T0901317 Does Not Lead to Lipid Accumulation in the Heart

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