

RESEARCH PAPER

Low dose of the liver X receptor agonist, AZ876, reduces atherosclerosis in APOE*3Leiden mice without affecting liver or plasma triglyceride levels

Correspondence

José WA van der Hooft, PhD,
TNO BioSciences, PO Box 2215,
2301 CE Leiden, the Netherlands.
E-mail: jose.vanderhoorn@tno.nl

*Both authors contributed
equally

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JWA van der Hooft^{1,2*}, D Lindén^{3*}, U Lindahl³, MEA Bekkers¹,
M Voskuilen¹, R Nilsson³, J Oscarsson³, EL Lindstedt³ and
HMG Princen¹

¹TNO BioSciences, Leiden, the Netherlands, ²Department of Cardiology, Leiden University
Medical Center, Leiden, the Netherlands, and ³AstraZeneca R&D, Mölndal, Sweden

BACKGROUND AND PURPOSE

Liver X receptor (LXR) agonists are atheroprotective but often induce hypertriglyceridaemia and liver steatosis. We investigated the effect of a novel high-affinity LXR activator, AZ876, on plasma lipids, inflammation and atherosclerosis, and compared the effects with another LXR agonist, GW3965.

EXPERIMENTAL APPROACH

APOE*3Leiden mice were fed an atherogenic diet alone or supplemented with either AZ876 (5 or 20 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) or GW3965 (17 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) for 20 weeks. Total cholesterol and triglyceride levels were measured using commercial kits. Plasma cytokines were determined by using bead-based multiplex suspension array kits with the Luminex technology. Atherosclerosis was assessed histochemically and lesion composition was assessed by immunohistochemical methods.

KEY RESULTS

Low-dose AZ876 had no effect on plasma or liver lipids, whereas high-dose AZ876 increased plasma triglycerides (+110%) and reduced cholesterol (-16%) compared with controls. GW3965 increased plasma triglycerides (+70%). Low-dose AZ876 reduced lesion area (-47%); and high-dose AZ876 strongly decreased lesion area (-91%), lesion number (-59%) and severity. In either dose, AZ876 did not affect lesion composition. GW3965 reduced atherosclerosis and collagen content of lesions (-23%; $P < 0.01$). High-dose AZ876 and GW3965, but not low-dose AZ876, reduced inflammation as reflected by lower cytokine levels and vessel wall activation.

CONCLUSIONS AND IMPLICATIONS

We have identified a novel LXR agonist that when given in a low dose inhibits the progression of atherosclerosis without inducing anti-inflammatory effects, liver steatosis or hypertriglyceridaemia. Therefore, the primary protective action of a low-dose AZ876 is likely to be an increased reverse cholesterol transport.

Abbreviations

ABC, ATP binding cassette transporter; ALT, alanine aminotransferase; apoE, apolipoprotein E; C4, 7 α -hydroxy-4-cholesten-3-one; CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; CT, cycle threshold; LXR, liver X receptor; RCT, reverse cholesterol transport; SMC, smooth muscle cell; SR-BI, scavenger receptor class B type I; SREBP1-c, sterol-response element-binding protein 1-c

Introduction

Atherosclerosis is a dynamic process driven by both inflammation and hyperlipidaemia, and hence treatment should target both aspects of this disease. The current therapies to treat atherosclerosis primarily target hyperlipidaemia. However, different studies have shown that a substantial residual cardiovascular risk remains, even with very aggressive reductions in levels of low-density lipoprotein (LDL)-cholesterol (Baigent *et al.*, 2005). Therefore, new therapies focusing on this unmet need are highly desirable.

The liver X receptors (LXR α or NR1H3 and LXR β or NR1H2; nomenclature follows Alexander *et al.*, 2009) are members of the nuclear receptor superfamily of transcription factors. It is now appreciated that both LXR isoforms function as intracellular sensors of cholesterol excess. LXR α is predominantly expressed in tissues and cells that play important roles in lipid homeostasis, such as the liver, intestine, adipose tissue and macrophages, while LXR β is expressed in many cell types (Repa and Mangelsdorf, 2000). The natural ligands for both LXR isoforms include oxidized derivatives of cholesterol (i.e. oxysterols) (Janowski *et al.*, 1996; Lu *et al.*, 2001). Synthetic high-affinity ligands have also been identified, such as T0901317 and GW3965 (Schultz *et al.*, 2000; Collins *et al.*, 2002). The activation of LXR induces numerous genes involved in cholesterol homeostasis, lipogenesis and reverse cholesterol transport (RCT), including those for the ATP binding cassette transporter A1 (ABCA1), ABCG1, ABCG5, ABCG8, scavenger receptor class B type I (SR-B1), cholesteryl ester transfer protein (CETP), apolipoprotein E (apoE), lipoprotein lipase and sterol-response element-binding protein 1-c (SREBP1-c) (Zelcer & Tontonoz, 2006; Rigamonti *et al.*, 2008). RCT is believed to be the main effect of LXR agonists in the prevention or treatment of atherosclerosis. ABCA1, ABCG1, apoE and SR-B1 are the principal proteins involved in RCT to promote cholesterol efflux from macrophage foam cells to high-density lipoprotein (HDL) particles for subsequent delivery of cholesterol to hepatocytes and enterocytes (Brunham *et al.*, 2006; Tall, 2008). In addition to their effects on cholesterol and lipid metabolism, the LXRs repress inflammatory actions of macrophages, by, for instance, interfering with the NF- κ B signalling (Rigamonti *et al.*, 2008).

LXR agonists have also been shown to cause hepatic steatosis and hypertriglyceridaemia in animals, primarily resulting from induction of the expression of hepatic SREBP1c (Repa *et al.*, 2000). These effects have hindered the develop-

ment of LXR agonists for human use until now. However, substantial interest remains and it is believed that more specific or more subtle LXR agonists may reduce atherosclerosis without demonstrating adverse effects (Rader, 2007; Tall, 2008; Kratzer *et al.*, 2009; Quinet *et al.*, 2009).

The aim of the present study was to compare the newly identified LXR agonist AZ876 (Figure 1) (Boström *et al.*, 2006) with GW3965 [a recognized selective LXR agonist with minimal effects on plasma and hepatic triglyceride levels (Joseph *et al.*, 2002; Miao *et al.*, 2004)], with regard to its effects on plasma and liver lipid levels and atherosclerosis development. We used female APOE*3Leiden transgenic mice, which are a well-established mouse model for hyperlipidaemia and atherosclerosis (van Vlijmen *et al.*, 1994). These mice have a lipoprotein profile similar to the profile of patients with familial dys- β -lipoproteinaemia in which the elevated plasma cholesterol and triglyceride levels are mainly confined to the very low-density lipoprotein (VLDL)/LDL-sized lipoprotein fraction. In contrast to other mouse models for dyslipidaemia and/or atherosclerosis (Zadelaar *et al.*, 2007), these mice respond in a similar manner to human patients when treated with human therapies for cardiovascular diseases, such as statins, cholesterol uptake inhibitors, calcium channel blockers, fibrates and angiotensin II receptor antagonists (Delsing *et al.*, 2001; 2003; Kleemann *et al.*, 2003; Verschuren *et al.*, 2005; Kooistra *et al.*, 2006; van der Hoorn *et al.*, 2007).

Methods

Binding and transactivation assays

Binding vectors for His-tagged protein production were prepared by inserting the ligand-binding domain cDNA of human LXR α (amino acids 205–447) in pET28 (Novagen, Gibbstown, NJ, USA) and the ligand-binding domain cDNA of LXR β (amino acids 216–461) in pET24D. Proteins were expressed in *Escherichia coli* and purified on Ni⁺ columns. Binding assays using LXR α and LXR β protein were run by adding reagents to Wallac Isoplate 1450–514. Briefly, each 96 plate well contained assay buffer (20 mM Tris pH 7.5, 80 mM NaCl, 2 mM dithiothreitol, 0.125% Chaps and 10% glycerol), 0.1 mg SPA beads (polylysine-coated yttrium silicate beads, RPNQ0010P, GE Healthcare, Piscataway, NJ, USA), LXR α (0.5 μ g) or LXR β (0.25 μ g), 30 nM ³H-ligand (Tularik T0901317, specific activity of 473 KqBq nmol⁻¹) and test compound in a 10-point

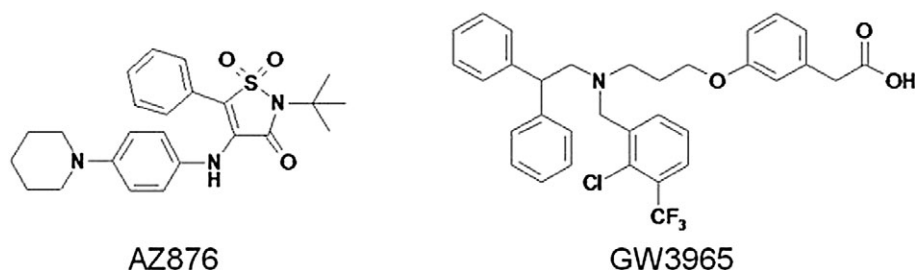


Figure 1

Chemical structures of AZ876 and GW3965.

dose-response dilution. The assay mixture was shaken gently for 2 h on a plate shaker after which the beads were allowed to settle for 1 hour before counting.

Transactivation vectors were prepared by inserting the ligand-binding domain cDNA sequences of human or mouse LXR α and LXR β in frame with the yeast Gal4 transcription factor DNA binding domain and the nuclear localization signal from the T-antigen of polyoma virus in the eucaryotic expression vector pSG5 (Stratagene, La Jolla, CA, USA). The ligand-binding domain cDNA of human LXR α and LXR β was the same as mentioned previously. The mouse sequence corresponded to amino acids 203–445 for LXR α and amino acids 201–446 for LXR β . The vectors were co-transfected with a pGL3 luciferase reporter plasmid containing a minimal SV40 promoter (Promega, Madison, WI, USA) and five copies of the UAS Gal4 recognition site into U2/OS osteosarcoma cells. Ligands were added as 10-point dose-response curves and then luciferase activity was measured after 48 h.

Macrophage RCT

All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of the Netherlands Organization for Applied Scientific Research (TNO) or by the Ethics Review Committee on Animal Experiments (Gothenburg region). The animals received food and water *ad libitum*. Body weight and food intake were monitored during the study. Macrophage RCT was determined essentially as described by Naik *et al.* (2006). J774A1 macrophages (obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen, DSMZ, Braunschweig, Germany) were cultured in RPMI supplemented with 10% fetal bovine serum and radiolabelled with 5 $\mu\text{Ci mL}^{-1}$ [^3H]cholesterol (specific activity 40–60 Ci/mmol, PerkinElmer, Waltham, MA, USA) together with 100 $\mu\text{g mL}^{-1}$ acetylated LDL (Intracel, Rockville, MD, USA) and 1% BSA (Sigma, St Louis, MO, USA) for 48 h. The J774A1 cells were then washed with phosphate-buffered saline (PBS) and resuspended in medium. The viability was estimated to be ~75% using Trypan blue. About 75% of the ^3H was found in the cholesteryl ester fraction of the cells before injection into the mice. Individually housed and body weight-matched 7-week-old C57BL/6J males (Harlan, the Netherlands) were treated with vehicle (amorphous nanoparticles with 3.4% dimethylacetamide), AZ876 (20 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$, ~9 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$), or GW3965 (34 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$, ~20 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) by oral gavage twice daily for 8 days. On day 6, labelled J774A1 cells (2.5×10^6 viable cells containing 28×10^6 dpm in 0.4 mL medium) were injected intraperitoneally. Faeces were collected between 24 and 48 h after macrophage injection. At termination (48 h after macrophage injection), the animals were anaesthetized with isoflurane (Forene, Abbot Scandinavia AB, Solna, Sweden), plasma was collected and the liver was collected after perfusion with PBS. Faeces (total) and liver (100 mg) were homogenized in PBS and total lipids were extracted from faeces and liver homogenates and plasma according to Folch *et al.* (1957). Free cholesterol and cholesteryl ester were separated using thin layer chromatography (hexane : diethylether : acetic acid 90:10:1). [^3H] in total extracted lipids, free cholesterol and cholesteryl ester were counted in a liquid scintillator and presented as percent dpm injected into the animal. The lipoprotein particles were separated using size-

exclusion high-performance liquid chromatography and the [^3H] was found in the HDL fraction (data not shown).

Study of treatments for atherosclerosis

Female heterozygous APOE*3Leiden transgenic mice (about 13 weeks of age, bred by TNO), on a C57bl/6 background and characterized by ELISA for human apoE (van Vlijmen *et al.*, 1994), were used. During a 3 week run-in period, all animals received a semi-synthetic Western-type diet containing 40.5% sucrose, 15% cacao butter, 0.50% (w/w) cholesterol and 0.33% v/w sunflower oil (necessary to dissolve the compounds). After matching into four groups, based on age, plasma cholesterol and triglyceride levels, the mice received Western-type diet either alone (control group) or supplemented with AZ876 in a low dose (5 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$; ~2 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) or high dose (20 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$; ~9 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) or with GW3965 (17 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$; ~10 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) (Joseph *et al.*, 2002). Initially, the high dose of AZ876 was 10 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$; however, after 4 weeks of treatment, GW3965 induced hypertriglyceridaemia but AZ876 (10 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) did not. Therefore, it was decided to increase the dose of AZ876 to 20 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$. Both compounds were provided by AstraZeneca (Mölnådal, Sweden). After 20 weeks, mice were killed and the hearts, aortic root, livers and small intestine (duodenum) were isolated.

Plasma lipid and lipoprotein analysis and inflammation markers

After a 4 h fasting period from 9 AM to 1 PM, blood was collected and EDTA plasma was obtained subsequently (Sarstedt, Nümbrecht, Germany) and lipoproteins were separated by fast protein liquid chromatography (FPLC; Westerterp *et al.*, 2006). Total cholesterol (No-1489437, Roche Diagnostics, Indianapolis, IN, USA) and triglyceride (1488872, Roche Diagnostics) levels were measured. Alanine aminotransferase (ALT) was determined spectrophotometrically using a Reflotron system (Roche Diagnostics). Plasma cytokines were determined by using bead based multiplex suspension array kits with the Luminex technology on a BioPlex (Bio-Rad, Hercules, CA, USA).

7 α -Hydroxy-4-cholesten-3-one (C4), lanosterol and β -sitosterol were analysed in serum by HPLC/MS/MS with atmospheric pressure photo ionization (Nilsson *et al.*, 2009). Plasma samples (50 μL) were extracted with isopropanol : heptane 4:1, in which the internal standards were dissolved and injected into the LC/MS/MS system. Samples were ionized in positive mode with multiple reaction monitoring. For C4, the transition at 401 m/z to 177 m/z was monitored. $^2\text{H}_7$ -7-ketocholesterol was monitored at m/z = 408 to 175. Lanosterol was monitored at transition 409.2 to 191.1, $^2\text{H}_3$ -lanosterol was monitored at transition 412.2 to 191.1, β -sitosterol was monitored at transition 397.2 to 161 and $^2\text{H}_7$ - β -sitosterol was monitored at transition 404.2 to 161 at CE = 22 eV. Samples were quantified by external standard calibration using a proxy matrix for calibration samples and deuterated internal standards as volume markers.

Faecal cholesterol and bile acids

Faeces were ground to powder in a mortar and then a 100 mg sample was shaken with 2 mL isopropanol/dioxan (1:1)

overnight. Total bile acids and cholesterol in the extracts were measured using commercial reagent systems [Bile Acids-L3K assay (Diagnostic Chemicals Limited, Mansfield, TX, USA) and Chol, Roche/Hitachi 12016630 122 (Roche Diagnostics)]. The assays were performed on a Cobas Mira Analyser (Hoffman-La Roche & Co., Basel, Switzerland).

Histological assessment of atherosclerosis

After the 20 week treatment period, the mice were killed. The hearts with aortic root were dissected, formalin fixed and embedded in paraffin. Serial cross-sections (5 µm thick, spaced 50 µm apart) throughout the entire aortic valve area were used for histological analysis. Sections were stained with haematoxylin-phloxine-saffron. For each mouse, four sections with intervals of 50 µm were used for quantification and qualification of the atherosclerotic lesions. For determination of the severity of atherosclerosis, the lesions were classified into five categories as described previously (Delsing *et al.* 2001; 2003; Verschuren *et al.* 2005): (i) early fatty streak; (ii) regular fatty streak; (iii) mild lesion; (iv) moderate lesion; and (v) severe lesion. The percentages of all lesions found in the respective categories were calculated. The total lesion area was calculated per cross-section. In each segment used for lesion assessment, the collagen content of the lesion was quantified morphometrically after Sirius Red staining and the smooth muscle cell (SMC) content after immunostaining with mouse anti-human α-actin (DAKO, Glostrup, Denmark), which cross reacts with mouse α-actin. The number of monocytes adhering to the endothelium was counted and the macrophage area was measured quantitatively after immunostaining with AIA31240 (Accurate Chemical and Scientific Corporation, Westbury, NY, USA). All analyses were performed by the same operator, using a double blind protocol.

RNA preparation and quantitative real-time PCR

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized from DNase treated total RNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR analysis was performed with an ABI Prism 7900 Sequence

Detection System using FAM and TAMRA labelled fluorogenic probes (Applied Biosystems). Expression data were normalized against mouse acidic ribosomal phosphoprotein P0 (m36B4). The relative expression levels were calculated according to the formula $2^{-\Delta CT}$, where ΔCT is the difference in cycle threshold (CT) values between the target and the m36B4 internal control. ABCA1 sequences; primer 5'-AAG GTTTCTTTGCTCAGATTGTC- 3', reverse primer 5'-TGCCA AAGGTGGCACA-3' and probe 5'-FAM-CCAGCTGTCTTT GTTTCATTGCC-TAMRA-3'. ABCG1 sequences; primer 5'-CCATGAATGCCAGCAGCTACT-3', reverse primer 5'-CA CTGACACGCACACGGACT-3' and probe 5'-FAM-TGCC GCAATGACGGAGCCC-TAMRA-3'. M36B4 sequences; primer 5'-GAGGAATCAGATGAGGATATGGGA-3', reverse primer 5'-AAGCAGGCTGACTTGGTTGC-3' and probe 5'-VIC-TCG GTC TCT TCG ACT AAT CCC GCC AA-TAMRA-3'.

Statistics

Values are expressed as mean ± SD. Comparisons between groups were made by Kruskal–Wallis ANOVA followed by Mann–Whitney U-test. *P* < 0.05 was considered significant.

Results

Identification and characterization of LXR agonist AZ876

In binding assays, AZ876 was 25-fold and 2.5-fold more potent than GW3965 on human (h)LXRα and hLXRβ respectively. In reporter transactivation assays, AZ876 was 196-fold and fivefold more potent than GW3965 on hLXRα and hLXRβ respectively. AZ876 was also more potent than GW3965 on mouse (m)LXRα (248-fold) and mLXRβ (10.5-fold) (Table 1). Thus, AZ876 is a more potent binder and activator of LXRα and LXRβ than GW3965. In addition, we have data showing that AZ876 is four- to sevenfold more potent than GW3965 on the expression of ABCA1 mRNA in hamster and human blood PMN cells, as determined following incubation with compounds *in vitro* (data not shown). Therefore, we believe both from human and mouse reporter assays as well as from hamster and human *in vitro* determination of ABCA1 gene

Table 1

Binding affinity (*K_i*) and transactivation potency (*EC*₅₀)

	LXRα Human K _i (µM)	EC ₅₀ (µM)	Mouse EC ₅₀ (µM)	LXRβ Human K _i (µM)	EC ₅₀ (µM)	Mouse EC ₅₀ (µM)
AZ876	0.007 ± 0.004 (<i>n</i> = 3)	0.006 ± 0.002 (<i>n</i> = 4)	0.005 ± 0.002 (<i>n</i> = 3)	0.011 ± 0.002 (<i>n</i> = 3)	0.073 ± 0.019 (<i>n</i> = 5)	0.065 ± 0.020 (<i>n</i> = 3)
GW3965	0.174 ± 0.069 (<i>n</i> = 5)	1.175 ± 0.472 (<i>n</i> = 15)	1.240 ± 0.306 (<i>n</i> = 3)	0.028 ± 0.014 (<i>n</i> = 5)	0.374 ± 0.171 (<i>n</i> = 22)	0.685 ± 0.158 (<i>n</i> = 3)

Scintillation proximity assay data showing the *K_i* values of GW3965 and AZ876 to displace [³H]-T0901317 bound to human LXRα or LXRβ protein. Reporter gene data showing *EC*₅₀ values of GW3965 and AZ876 from U2/OS cells co-transfected with a luciferase reporter gene containing Gal4 DNA-binding sites and Gal4 chimeric LXRα or LXRβ cDNA. Values are means or mean ± SD based on the indicated number of dose-responses (3–4 wells per dose-response).

Table 2

In vivo macrophage reverse cholesterol transport

	Control	AZ876 (20 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$)	GW3965 (34 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$)
Plasma lipids (% dpm inj.)			
[³ H]Total extracted	1.275 \pm 0.240	2.229 \pm 0.613**	1.689 \pm 0.539
[³ H]Free cholesterol	0.243 \pm 0.069	0.487 \pm 0.199**	0.362 \pm 0.095*
[³ H]Cholesteryl ester	0.686 \pm 0.148	1.550 \pm 0.686*	0.966 \pm 0.354
Liver lipids (% dpm inj.)			
[³ H]Total extracted	2.882 \pm 1.883	2.177 \pm 0.574	2.029 \pm 0.619
[³ H]Free cholesterol	1.981 \pm 0.710	1.754 \pm 0.451	1.748 \pm 0.518
[³ H]Cholesteryl ester	0.770 \pm 1.161	0.330 \pm 0.099	0.238 \pm 0.071
Faecal lipids (% dpm inj.)			
[³ H]Total extracted	0.603 \pm 0.141	1.17 \pm 0.893*	0.737 \pm 0.298
[³ H]Free cholesterol	0.214 \pm 0.068	0.632 \pm 0.570**	0.353 \pm 0.120*
[³ H]Cholesteryl ester	0.046 \pm 0.013	0.044 \pm 0.012	0.039 \pm 0.006

Body weight-matched C57BL/6J males were treated with AZ876 (20 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$, \approx 9 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$), GW3965 (34 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$, \approx 20 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) or vehicle (control) by oral gavage twice daily for 8 days. On the sixth day, mice were injected intraperitoneally with cholesterol-enriched [³H]cholesterol-labeled J774A1 macrophages. [³H]Total extracted lipids, [³H]free cholesterol and [³H]cholesteryl esters were determined in plasma and livers after 48 h and in faeces 24–48 h after injection of labelled J774A1 cells. Values are mean \pm SD, $n = 7$ –8, * $P < 0.05$, ** $P < 0.01$, significantly different from control.

expression data that AZ876 is more potent than GW3965 on RCT genes. Also, AZ876 was highly selective with respect to other nuclear hormone receptors, including retinoid X receptor, farnesoid X receptor, thyroid hormone receptor (TR) α or TR β , when tested in agonist mode in fluorescence resonance energy transfer assays (data not shown).

We also determined the effect of AZ876 and GW3965 on *in vivo* RCT by following the appearance of radiolabelled lipids in plasma, liver and faeces after injecting radiolabelled J774A1 macrophages into C57Bl/6J mice (Table 2). AZ876 (20 $\mu\text{mol}\cdot\text{kg}^{-1}$) increased [³H]total lipids by 75%, [³H]free cholesterol by 100% (both $P < 0.01$) and [³H]cholesteryl ester by 126% ($P < 0.05$) in plasma compared with vehicle controls. Furthermore, AZ876 increased [³H]total lipids recovered from the faeces by 94% ($P < 0.05$) and [³H]free cholesterol by 195% ($P < 0.01$) as compared with vehicle control. GW3965 (34 $\mu\text{mol}\cdot\text{kg}^{-1}$) increased macrophage RCT in accordance with the study from Naik *et al.* (2006) as demonstrated by increased levels of [³H]free cholesterol in plasma and faeces, by 49 and 65% (both $P < 0.05$), respectively, compared with vehicle controls. Collectively, these data show that AZ876 is a potent activator of the LXRs which is paralleled by a marked effect on *in vivo* RCT.

Effect of AZ876 and GW3965 on plasma lipid levels

The effect of both compounds on plasma lipid levels was investigated in hyperlipidaemic APOE*3Leiden mice. The mice were fed a Western-type diet, to induce hyperlipidaemia, giving on average plasma cholesterol levels of 18.2 \pm 3.9 mmol L^{-1} and triglyceride levels of 2.0 \pm 0.4 mmol L^{-1} in the control group (Figure 2A,B). AZ876 in low dose

(5 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) tended to decrease cholesterol levels by 12% [not significant (NS)], whereas it did not affect triglyceride levels. High-dose AZ876 (20 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) significantly decreased total cholesterol levels by 16% ($P < 0.05$) and increased triglyceride levels by 110% ($P < 0.001$). GW3965 tended to decrease cholesterol levels by 12% (NS) and increased plasma triglycerides by 70% ($P < 0.001$). The reduction in plasma cholesterol was confined to the VLDL fractions as measured after plasma separation by FPLC. Large HDL-1 particles in the lipoprotein profile were observed in the groups of mice treated with 20 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ AZ876 or with GW3965, but not in the mice treated with the lower dose of AZ876 (5 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) (Figure 2C). This effect is likely to be related to the increased *abca1* mRNA expression in the intestine in the two groups (+410% in the high AZ876 group and +330% in the GW3965 group, both $P < 0.01$) as intestinal ABCA1 directly contributes to the HDL-raising effect of LXR agonists (Brunham *et al.*, 2006). Supporting this idea, we found that the aortic mRNA expression of both *abca1* (+101% and +120%, both $P < 0.05$) and *abcg1* (+164%, $P < 0.001$ and +154%, $P < 0.05$) were increased in these groups (Figure 3).

Effect of AZ876 and GW3965 on synthesis, intestinal uptake and faecal output of cholesterol

We determined whether an altered intestinal cholesterol uptake, changed cholesterol synthesis or faecal output of cholesterol or bile acids could have contributed to the changes in plasma cholesterol levels. Serum levels of lanosterol, reflecting the cholesterol synthesis (Bjorkhem *et al.*, 1987), were increased in the high-dose AZ876 (+310%,

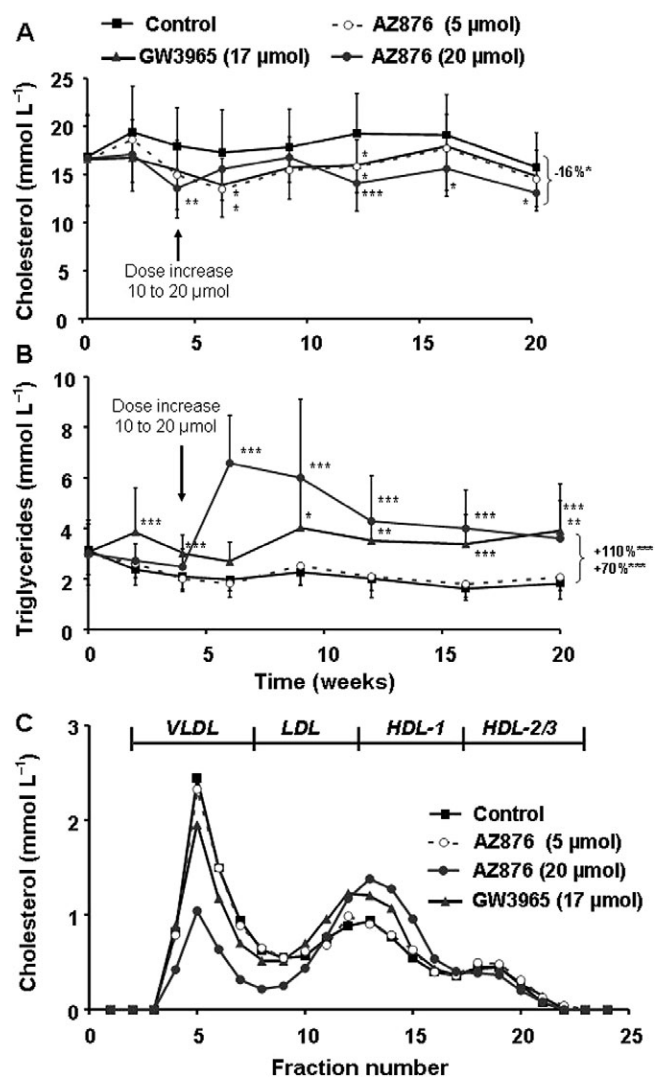


Figure 2

APOE*3Leiden mice were treated for 20 weeks with a Western-type diet alone (control) or supplemented with AZ876 (5 or 20 μmol·kg⁻¹·day⁻¹), or with GW3965 (17 μmol·kg⁻¹·day⁻¹). The plasma cholesterol levels (A) and triglyceride levels (B) over 20 weeks are shown. Lipoproteins were separated by fast protein liquid chromatography and cholesterol profiles were measured in pooled plasma per group at the end of the study (C). Dose of AZ876 was increased from 10 to 20 μmol 4 weeks after treatment as indicated (arrow). Values are mean ± SD. *n* = 15, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, significantly different from control.

P < 0.01) and GW3965 (+141%, *P* < 0.001), but not in the low AZ876 group (+29%, NS).

Food intake and body weight showed no difference between the groups, reflecting a similar cholesterol intake. In addition, β-sitosterol was measured as a marker for intestinal cholesterol uptake. β-Sitosterol levels were not affected by any of the treatments, indicating no change in intestinal cholesterol uptake. We then investigated the faecal excretion of cholesterol and bile acids. Faecal cholesterol output was 0.12 ± 0.02 mmol/100 g bw/day in the control group and unchanged by either LXR agonist. The faecal bile acid output

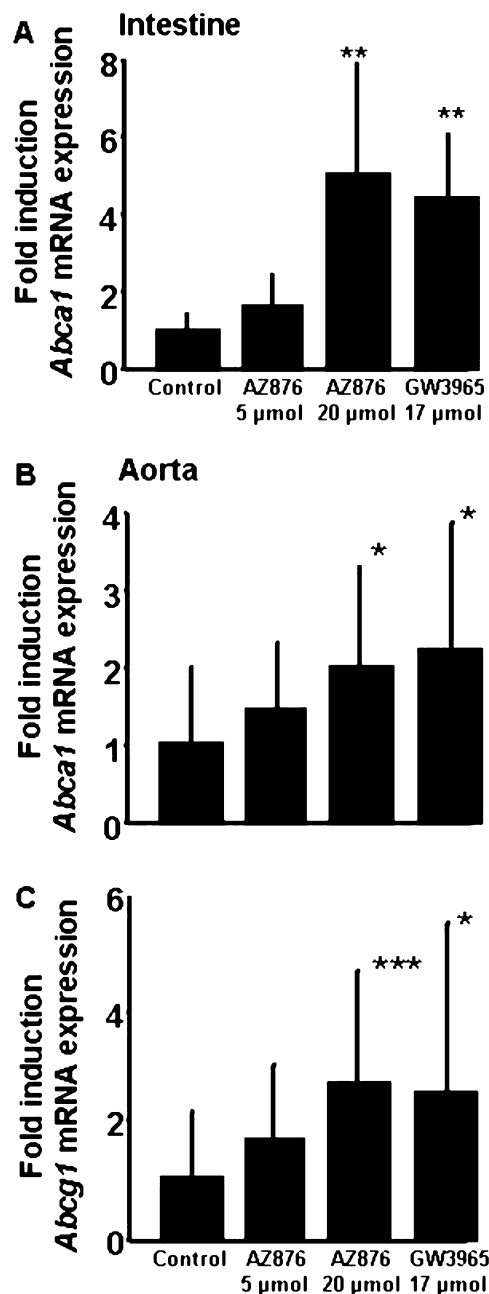


Figure 3

APOE*3Leiden mice were treated for 20 weeks with a Western-type diet alone (control) or supplemented with AZ876 (5 or 20 μmol·kg⁻¹·day⁻¹), or with GW3965 (17 μmol·kg⁻¹·day⁻¹). Intestinal mRNA expression of *abca1* (A) and aortic mRNA expression of *abca1* (B) and *abcg1* (C) are shown. Values are mean ± SD. *n* = 15, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, significantly different from control.

was also unchanged (11.1 ± 2.4 mmol/100 g bw/ day in the control group), which was in line with the unaffected serum levels of the marker of bile acid biosynthesis, 7α-hydroxy-4-cholesten-3-one (C4). In summary, the LXR agonists induce cholesterol synthesis, but it was not possible to detect changes in cholesterol absorption or losses as demonstrated

Table 3

Effects of AZ876 and GW3965 on liver weight, triglycerides and plasma ALT levels

	Control	AZ876 (5 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$)	AZ876 (20 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$)	GW3965 (17 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$)
Liver weight (g)	1.43 \pm 0.22	1.34 \pm 0.22	1.76* \pm 0.35	1.37 \pm 0.16
Triglycerides (g/100 g tissue)	9.7 \pm 1.5	10.7 \pm 1.6	14.8** \pm 3.5	11.3 \pm 2.3
ALT (U/L)	105 \pm 19	94 \pm 14	225** \pm 43	87 \pm 7

Plasma alanine aminotransferase (ALT) levels as measured in plasma pooled per group. Values are mean in time ($n = 5$ per group) \pm SD, * $P < 0.05$, ** $P < 0.01$, significantly different from control.

by no change in markers of cholesterol absorption or faecal loss of cholesterol or bile acids.

Effect on liver triglyceride content

LXR agonists are notorious for their induction of hepatosteatosis and we therefore measured liver weight and triglyceride content. The Western-type diet induced a slight steatosis in the control group (Table 3). Treatment with 5 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ AZ876 or GW3965 did not affect liver weight and triglyceride content, whereas the high dose of AZ876 (20 $\mu\text{mol}\cdot\text{kg}^{-1}$) increased both liver weight (+29%, $P < 0.05$) and triglyceride content (+53%, $P < 0.01$). Concomitantly, plasma ALT levels, as a marker for liver damage, were on average 114% increased ($P < 0.01$) after treatment with the higher dose of AZ876, as measured in pooled samples per group. No ALT increase was observed in the 5 $\mu\text{mol}\cdot\text{kg}^{-1}$ AZ876- and GW3965-treated animals (Table 3).

Effect of GW3965 and AZ876 on atherosclerosis

In order to determine the effect of both LXR agonists on atherosclerosis development, we measured the number of lesions and the total lesion area. We also phenotyped the severity of these lesions in the aortic root after a 20 week treatment period. About half (54 \pm 24%) of the lesions in the control group (Figure 4) were severe types IV–V lesions. This figure also shows that 5 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ AZ876 did not affect lesion number, whereas it decreased lesion area by 47% ($P < 0.05$) and tended to decrease the amount of severe lesions (36 \pm 24%, $P = 0.056$). AZ876 (20 $\mu\text{mol}\cdot\text{kg}^{-1}$) markedly reduced atherosclerosis development with regard to lesion number (–59%), lesion area (–91%) and the abundance of severe lesions (17 \pm 23%, all $P < 0.001$). GW3965 was more potent in inhibiting atherosclerosis than the low dose of AZ876, resulting in reductions in the number of lesions (by 38%, $P < 0.05$) and lesion area (by 74%, $P < 0.001$) and less severe lesions (28 \pm 21%, $P < 0.01$) as compared with control. We observed no effect of 5 $\mu\text{mol}\cdot\text{kg}^{-1}$ AZ876 on the amount of undiseased segments (14 \pm 21% in the control). However, 20 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ AZ876 and GW3965 clearly increased the amount of undiseased segments (to 58 \pm 23%, $P < 0.001$ and 44 \pm 28%, $P < 0.01$, respectively). Both high-dose AZ876 and GW3965 were significantly ($P < 0.001$ and $P < 0.05$, respectively) more potent in preventing lesion development than the low-dose AZ876 treatment.

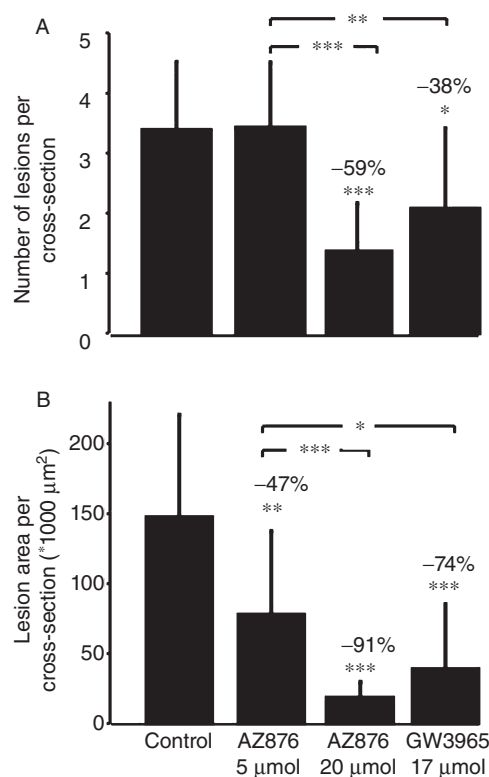


Figure 4

APOE*3Leiden mice were treated for 20 weeks with a Western-type diet alone (control) or supplemented with AZ876 (5 or 20 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) or GW3965 (17 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$). The amount of lesions in the aortic root was counted (A) and the lesion area was measured (B). Values are mean \pm SD. $n = 15$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significantly different from control.

We then assessed the lesion composition by measuring the collagen and the SMC content of the lesions, both considered to stabilize the lesions, and the amount of macrophages, known to be a destabilizing component in the lesions (Delsing *et al.*, 2001; Libby, 2002). As shown in Figure 5, treatment with AZ876 in either dose did not affect the collagen or SMC content, relative to values in the control lesions. GW3965, however, reduced the collagen content by 23% ($P < 0.01$) as compared with control, resulting

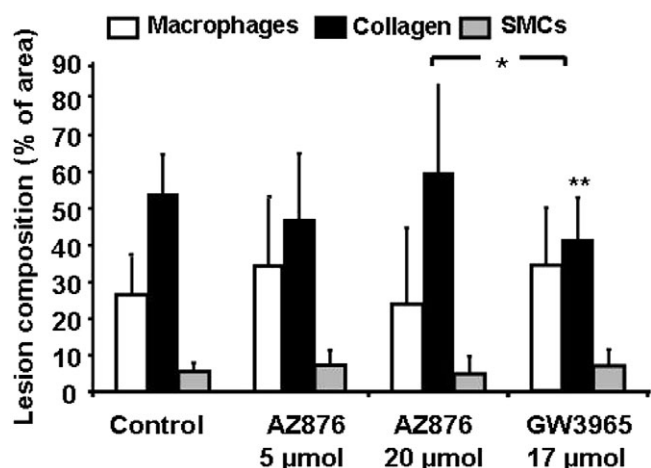


Figure 5

The effect of 5 or 20 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ AZ876 or GW3965 (17 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) on lesion composition was assessed by measuring the macrophage, collagen and smooth muscle cell (SMC) area, all calculated as percentage of the total lesion area. Values are mean \pm SD. $n = 15$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significantly different from control.

in significantly less collagen content than in the 20 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ AZ876 group, without affecting the SMC content. Treatment with either dose of AZ876 or GW3965 did not significantly affect the macrophage content (Figure 5). In summary, GW3965 induced a less stable lesion phenotype as compared with the control, whereas AZ876 in either dose did not affect the lesion composition.

As a functional parameter for vessel wall inflammation, we also investigated the numbers of monocytes adhering to the activated endothelium of the aortic root, which is considered the first step in lesion development. A representative image of adhering monocytes is presented in Figure 6A. The summary data (Figure 6B) show that the number of adherent macrophages was reduced by 72% upon treatment with 20 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ AZ876 and by 53% in the GW3965-treated mice. These anti-inflammatory effects of both LXR agonists were confirmed by plasma analysis of inflammatory cytokines TNF- α , IL-1 β and IL-6 (Table 4), which were decreased upon treatment with 20 $\mu\text{mol}\cdot\text{kg}^{-1}$ AZ876 and with GW3965. Low-dose AZ876 did not affect monocyte adherence or plasma cytokine levels (Figure 6B and Table 4).

Discussion

In this study, we evaluated the effect of the novel LXR agonist AZ876 on plasma lipid levels and atherosclerosis in hyperlipidaemic APOE*3Leiden transgenic mice. AZ876 and GW3965 both markedly inhibited atherosclerosis development, whereas AZ876 induced a more stable lesion phenotype. Additionally, AZ876 showed no adverse effects when given in low dose, pointing to dose-dependency of the effects induced by AZ876.

The results of the present study confirm the findings of previous studies in APOE*3Leiden (Grefhorst *et al.*, 2002;Ver-

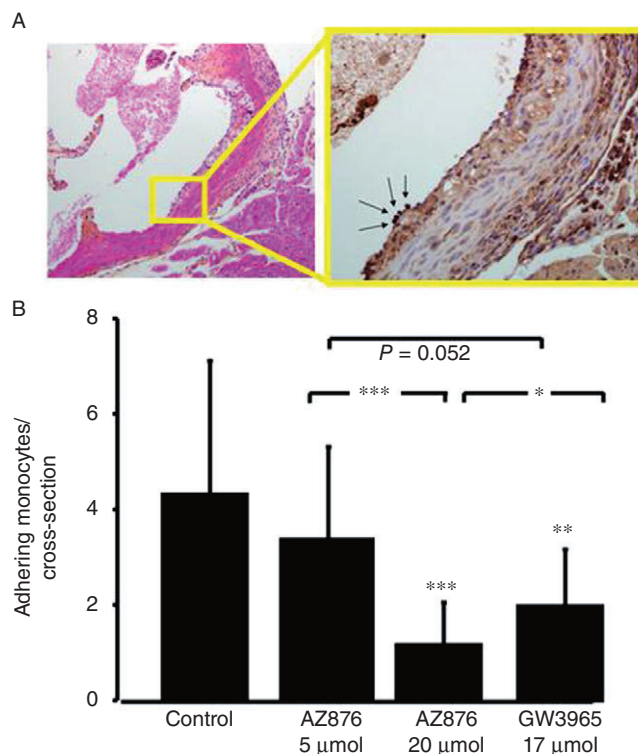


Figure 6

To investigate the effect of 5 or 20 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ AZ876 or GW3965 (17 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) on vessel wall inflammation, monocytes were stained in the aortic root (A). The number of monocytes adhering to the endothelium was counted in each animal (B). Values are mean \pm SD. $n = 15$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significantly different from control.

schuren *et al.*, 2009), LDLr^{-/-} (Terasaka *et al.*, 2003) and apoE^{-/-} (Dai *et al.*, 2007) mice with another LXR agonist, T0901317, with regard to elevation of plasma triglyceride and HDL levels and reduction of atherosclerosis. In wild-type mice, GW3965 (20–100 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) has been reported to be a tissue- and gene-selective modulator of LXR activity with less lipogenic effects than T0901317 after a 3 day treatment (Miao *et al.*, 2004). Also in LDLr^{-/-} mice, GW3965 (10 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) did not affect plasma or liver lipid levels when administered for 8 weeks (Quinet *et al.*, 2009). In contrast to these latter reports, we showed that in a more sensitive model of hyperlipidaemia, the APOE*3Leiden mouse, even a relatively low dose of GW3965 given for 20 weeks (17 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$, ~10 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) increased plasma triglyceride levels. The low dose of 5 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ AZ876 did not affect plasma triglycerides; however, the high dose of 20 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ AZ876 did induce hypertriglyceridaemia indicative of a dose-dependent effect. Next to the undesired induction of hypertriglyceridaemia, GW3965 and 20 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ AZ876 increased HDL levels, thereby resembling findings described for T0901317 and fenofibrate-treated APOE*3Leiden mice (Kooistra *et al.*, 2006;Verschuren *et al.*, 2009), as reflected by the appearance of a large cholesteryl ester-rich HDL-1 particle in the lipoprotein profile, containing mainly the apoE, and to

Table 4

Effects of AZ876 and GW3965 on plasma cytokines

	Control	AZ876 (5 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$)	AZ876 (20 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$)	GW3965 (17 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$)
TNF- α (pg mL $^{-1}$)	1110 \pm 1294	955 \pm 865	521* \pm 454	362** \pm 322
IL-1 β (pg mL $^{-1}$)	191 \pm 141	213 \pm 166	114* \pm 52	94** \pm 44
IL-6 (pg mL $^{-1}$)	124 \pm 130	102 \pm 68	65 \pm 47	43** \pm 18

Plasma levels of cytokines as measured by luminex assay. Values are mean \pm SD, * P < 0.05, ** P < 0.01, significantly different from control.

a minor extent apoB and no apoAI (Gruen *et al.*, 2005). The appearance of this large HDL-1 particle upon LXR agonist treatment is specific for species without CETP and may have atheroprotective effects by supporting apoAI-independent cholesterol efflux (Jiang *et al.*, 1992). In species containing CETP, like hamsters and cynomolgus monkeys (Groot *et al.*, 2005; Quinet *et al.*, 2009) and APOE*3Leiden.CETP transgenic mice (PCN Rensen and HMG Princen, unpubl. data), this HDL is absent via the action of CETP. In our study, the increased HDL levels were in accordance with an increase in intestinal *abca1* mRNA expression in the GW3965 and 20 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ AZ876 group, as intestinal ABCA1 directly contributes to the HDL raising effect of LXR agonists (Brunham *et al.*, 2006; Tang *et al.*, 2008).

Additionally, we observed increased lanosterol levels, suggesting an increased cholesterol synthesis. The most likely explanation for this finding is an increased cholesterol loss. However, we could not detect any signs of increased cholesterol loss by measuring biomarkers for cholesterol uptake (sitosterol) or bile acid synthesis (C4) or changed cholesterol or bile acid content in faeces. Another explanation could be the down-regulation of the cholesterologenic enzyme lanosterol 14 α -demethylase (CYP51A1) by LXR activation, which in turn leads to an accumulation of lanosterol. However, as opposing effects of distinct ligands (natural oxysterols vs. synthetic ligands) have been described for the regulation of CYP51A1 expression, this remains unclear (Wang *et al.*, 2008).

Whereas the changes in lipoprotein profiles (reduction in VLDL and rise in HDL) *per se* are anti-atherogenic, we also observed reductions in circulating levels of the cytokines TNF α , IL-1 β and IL-6, reflecting reduced inflammation brought about by an equimolar dose of AZ876 and GW3965. Furthermore, we showed that both agonists are potent inducers of RCT *in vivo*. This was studied in male C57BL/6 mice on standard chow diet in order to compare results with already published GW3965 data (Naik *et al.*, 2006). The percent of radioactivity from injected 3H-labelled cholesterol found in plasma, liver and faeces in the present study is similar to the latter study. Importantly, the [^3H]-radioactivity detected in blood was found in the HDL fraction. Thus, the radioactivity detected in blood most likely did not derive from circulating macrophages but involved an active transport of labelled cholesterol in HDL. It can only be speculated that most of the labelled cholesteryl ester still resides in macrophages within the peritoneal cavity after 48 h. In addition to this *in vivo* RCT experiment, we have in-house data to show that AZ876

reduces atherosclerosis in male apoE-deficient mice (data not shown). Moreover, LXR activation has been found by other groups to reduce atherosclerosis in both male and female apoE-deficient mice (Kratzer *et al.*, 2009). Thus, although not shown in the present study, there are data to support anti-atherogenic effects of LXR agonists including AZ876 in both male and female mice.

We then investigated the effects of the compounds on atherosclerosis development. Both GW3965 and AZ876 at equimolar doses were very potent in reducing all parameters of atherosclerosis (i.e. lesion number, area and severity). The low dose of AZ876 (5 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) reduced the lesion area and tended to reduce abundance of severe lesions without affecting the number of lesions. Additionally, and in line with the reduction in plasma cytokine levels, both GW3965 and high-dose AZ876 decreased the amount of monocytes adhering to the vessel wall, which is considered as a functional parameter for vessel wall inflammation. Looking into further detail into lesion composition, we observed a discrepancy between the effect of 17 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ GW3965 and of the similar dose of 20 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ AZ876. Treatment with GW3965 appeared to induce a less stable lesion phenotype than the equimolar dose of AZ876, as reflected by lower collagen content.

In line with our data in APOE*3Leiden mice, Joseph *et al.* (2002) reported that GW3965 prevented atherosclerosis development in LDLr $^{-/-}$ and apoE $^{-/-}$ mice, which was attributed mainly to a direct effect on cholesterol efflux from macrophages in the vessel wall. The latter study, unfortunately, did not show data on the lesion composition. Extending these findings, the same group demonstrated that GW3965 also has anti-inflammatory effects in the vessel wall, which also may have contributed to the observed atheroprotective effect. Our study in APOE*3Leiden mice confirms the anti-inflammatory effect of GW3965 as reflected by reduced levels of plasma cytokines and a decreased adherence of monocytes.

In conclusion, we have shown that, in contrast to the higher dose of AZ876, low-dose AZ876 did not adversely affect plasma and liver lipid levels, the lipoprotein profile and did not reduce inflammation, but still inhibited lesion development. This indicates that the presence of the large HDL-1 particle and anti-inflammatory effects, as observed after high-dose AZ876 treatment, is not the sole explanation of its anti-atherosclerotic effect. AZ876 (5 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) did not affect the amount of adhering monocytes and the number of lesions, and the amount of undiseased segments were unaffected. Thus, the compound did not inhibit the onset of

lesion development; instead it inhibited progression of the lesion area. As we showed that AZ876 is a very potent inducer of RCT *in vivo*, we suggest that this reduced lesion area is primarily due to an increased cholesterol efflux from the lesions by RCT. This idea is supported by the increased expression of ABCA1 and ABCG1 mRNA in the aorta in animals treated with the high dose of AZ876. Although the low dose only tended to increase the expression of these genes, it is possible that, over longer periods of time, such small changes might result in a physiologically relevant effect on RCT to influence progression of atherosclerosis.

Our data demonstrate the feasibility of identifying LXR agonists that inhibit the progression of atherosclerosis and, depending on the dose, without having an effect on inflammation and without inducing the detrimental lipogenic effects in liver and plasma. Identification of LXR modulators with a greater separation between beneficial and adverse effects remains an important challenge for the development of such agents for the future treatment of atherosclerosis.

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Conflicts of interest

DL, UL, RN, JO and ELL are employees of AstraZeneca R&D, Sweden.

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