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Brief Article

Discovery of 6-(4-{[5-Cyclopropyl-3-(2,6-dichlorophenyl)isoxazol-4-yl]methoxy}piperidin-1-yl)-1-methyl-1H-indole-3-carboxylic Acid: A Novel FXR Agonist for the Treatment of Dyslipidemia

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Discovery of 6-(4-{[5-Cyclopropyl-3-(2,6-dichlorophenyl)isoxazol-4-yl]methoxy}piperidin-1-yl)-1-methyl-1H-indole-3-carboxylic Acid: A Novel FXR Agonist for the Treatment of Dyslipidemia

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KEYWORDS. Farnesoid X receptor, FXR, nuclear hormone, agonist, piperidine, indole, dyslipidemia, atherosclerosis, diabetes.

ABSTRACT: The farnesoid X receptor (FXR) is a member of the 'metabolic' subfamily of nuclear receptors. Several FXR agonists have been reported in the literature to have profound effects on plasma lipids in animal models. In our efforts to discover novel and effective therapies for dyslipidemia and atherosclerosis we have developed a series of potent FXR agonists that robustly lower plasma LDL and vLDL in LDLr-/- mice. To this end the novel piperidinylisoxazole system LY2562175 was discovered. This molecule is a potent and selective FXR agonist in vitro and has robust lipid modulating properties lowering LDL and triglycerides while raising HDL in preclinical species. The pre-clinical ADME properties of LY2562175 were consistent with enabling once daily dosing in humans and it was ultimately advanced to the clinic for evaluation in humans. The synthesis and biological profile of this molecule is discussed.

INTRODUCTION

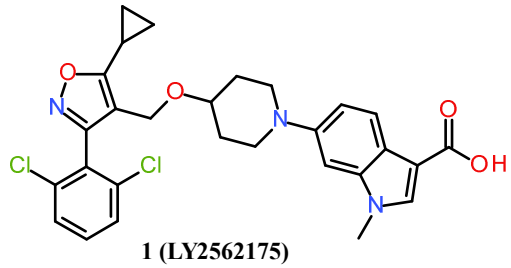
The farnesoid X receptor (FXR) is a member of the 'metabolic' subfamily of nuclear receptors. The discovery of bile acids as natural physiological ligands for FXR and the development of potent FXR-selective synthetic agonists illuminated the potential use of FXR agonists for treatment of chronic hepatitis, cholestasis, liver fibrosis and atherosclerosis.[1-3] The first evidence illustrating the importance of FXR in regulation of lipid homeostasis came from mice harboring a deletion construct of the FXR/BAR gene. When maintained on a 1% cholesterol diet, hepatic cholesterol and triglyceride contents were greater in FXR/BAR null versus wild-type mice. When maintained on chow diet, serum total cholesterol, phospholipid, triglyceride, and cholesterol ester levels for FXR/BAR null mice were significantly greater than those for wild-type mice. Thus, disruption of FXR/BAR gene results in a potentially proatherogenic serum lipoprotein profile that is exacerbated by increased dietary cholesterol and the evidence from the FXR/BAR null mice suggests that an FXR agonist may provide therapeutic benefit for dyslipidemia.[4]

Researchers from GlaxoSmithKline were first to report in vivo activity of a potent, synthetic FXR agonist, GW4064. When administered to Fischer rats, GW4064 elevated circulating HDL levels and reduced triglycerides.[5] Since this report, GW4064 has been successfully utilized as a research tool for exploring FXR mechanism of action and pharmacology.

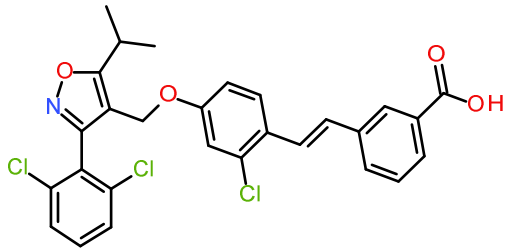
Improvements in dyslipidemia and glucose homeostasis in disease-state animal models following FXR agonist treatment suggest beneficial outcomes of FXR agonists for the indication of atherosclerosis. The pathological consequences of the loss of FXR function on the risk and severity of atherosclerosis was first tested when FXR null mice were crossed onto the atherogenic ApoE null background.[6] Loss of functional FXR on the ApoE null background led to further increases of total blood cholesterol and triglyceride levels as well as increased VLDL and LDL in the ApoE null model. High fat/high cholesterol (HF/HC) feeding in the FXR null x ApoE null mice caused the most severe effects on hepatic lipid accumulation, focal necrosis, and inflammatory gene expression. These phenotypic outcomes were associated with the most severe degree of atherosclerotic lesion formation and the lowest survivability in the HF/HC-fed FXR null x ApoE null mice as compared with the control genotypes.[7] Based on these results, FXR ap-

pears to play a protective role in managing risk factors that contribute to the development of atherosclerosis.

In our efforts to discover novel and effective therapies for dyslipidemia we have developed a series of potent FXR agonists that robustly lower plasma LDL and vLDL in LDLr-/- mice. To this end the novel piperidinylisoxazole system LY2562175 was discovered. This molecule is a potent and selective FXR agonist in vitro and has robust lipid modulating properties lowering LDL and triglycerides while raising HDL in preclinical species. LY2562175 also has ADME properties consistent with enabling once daily dosing and it was advanced to clinical evaluation in humans.



1 (LY2562175)



GW4064

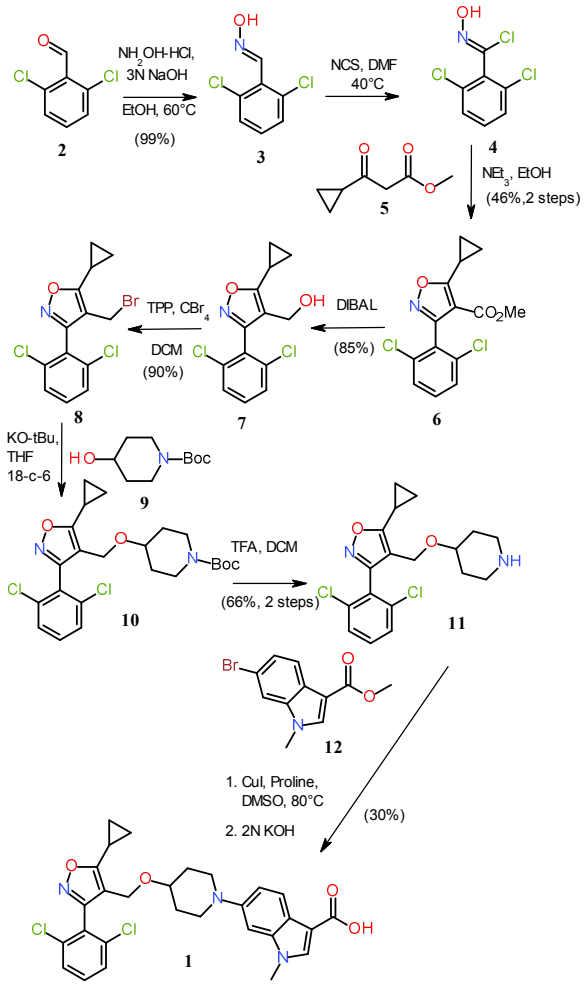
Figure 1. Structures of FXR agonists **1** (LY2562175) and GW4064.

RESULTS AND DISCUSSION

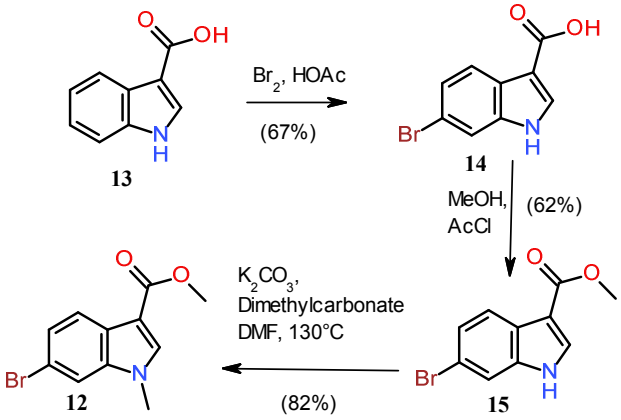
Chemistry. The synthesis of compound **1** was achieved in 9-linear steps as the longest sequence and is outlined in **Scheme 1**. Initially the oxime **3** was formed by condensing ammonium hydroxide with the commercially available aldehyde **2.** Clorination of **3** with NCS provided cloroxime **4** which was subjected to cyclization with the beta-keto ester **5** under mildly basic conditions to yield the desired isoxazole system **6.** Reduction of the ester (DIBAL) was followed by bromination of the resulting alcohol **7** to form the isoxazolyl-bromide system **8**. Reaction of **8** with boc-4-hydroxypiperidine was done under basic conditions followed by deprotection to form the coupling partner **11**. Coupling of **11** with the protected indole species **12** under copper/proline catalyzed cross-coupling conditions provided the penultimate intermediate as a methyl ester**.** Hydrolysis of the ester resulted in formation of the desired product **1** in ~7% overall yield.

The intermediate indole **12** was prepared as shown in **Scheme 2**. Commercially available indole-3-carboxylate **13** was brominated and esterified to form **15** under standard conditions. The indole nitrogen was then methylated to yield the requisite intermediate **12**.

Scheme 1. Synthesis of compound 1 (LY2562175).



Scheme 2. Synthesis of indole intermediate 12.



Biology and Pharmacology. The effect of **1** on a functional measure of FXR transcription transactivation activity was measured using co-transfected HEK293 cells, the human FXR cDNA, and an FXRE-luciferase reporter. The ability of the

compound to modulate the secondary structure of the FXR ligand binding domain to enable recruitment of a transcriptional coactivator was measured using purified recombinant FXR ligand binding domain protein and the nuclear receptor interaction domain of the co-activator protein, SRC-1, and the AlphaScreen technology **(Table 1**). LY2562175 promotes transcriptional activation of human FXR in a cell-based co-transfection assay with an EC50 of 193 nM (geometric mean) and demonstrates 'partial agonist-type' efficacy since the percent activation as compared to a full FXR agonist is approximately 41.3% efficacy relative to GW4064 a full agonist. LY2562175 promotes recruitment of a peptide from the nuclear receptor interaction domain of the co-activator SRC-1 with a relative EC50 of 121 nM and 93.5% efficacy as compared to GW4064.

Table I. FXR Agonist LY2562175 in vitro Profile.

Compound	hFXR FXRE Ag*		hFXR\hSRC1 CoAR 25uM**	
	Relative EC50 (nM)	% Relative Efficacy	Relative EC50 (nM)	% Relative Efficacy
2562175	193	41	121	93
GW4064	373	100	220	100

*FXR transcriptional potency and efficacy of LY2562175 in the HEK293 co-transfection assay. HEK293 cells were co-transfected with a human FXR expression plasmid and 2XIR-1-luciferase reporter plasmid. Determination of LY2562175 potency and % efficacy was established as compared to the full agonist, GW4064. **Recombinant FXR LBD protein was co-incubated with the nuclear receptor interaction domain of SRC-1. Interaction between the proteins was measured using AlphaScreen technology. Determination of LY2562175 potency and % efficacy was established as compared to the full agonist, GW4064.

Nuclear receptor selectivity was determined using a panel of nuclear receptor co-transcriptional activation assays and biochemical ligand binding assays. LY2562175 demonstrates selectivity for FXR since it does not promote transcriptional activation of other members of the nuclear receptor superfamily, such as glucocorticoid receptor, androgen receptor, mineralocorticoid receptor and progesterone receptor (EC50 > 10000 nM). Thus, LY2562175 is a potent agonist of human FXR both in biochemical assays and in cells and demonstrates selectivity as compared to other members of the nuclear receptor superfamily, including GR, AR, MR and PR.

To assess the potency and efficacy of the FXR agonist LY2562175 in vivo, studies were conducted in 8 week old, male LDLR null mice fed a 'western' diet. Animals were allowed to acclimate to the high fat/high cholesterol chow TD88137 (containing 0.15% cholesterol and 42% fat) for two weeks prior to the study. Animals were divided into groups of six and dosed once daily for one week by gavage with solutions of LY2562175 in situ sodium salt or with vehicle (5 % Solutol, 5% EtOH, 1% wt/v CMC) at a dose volume of 5 mL/kg. On the seventh day, animals were bled by cardiac puncture under anesthesia with CO2. Serum was prepared from individual animals for determination of cholesterol and triglycerides by enzymatic analysis using a Hitachi 912 Clinical Chemistry Analyzer with Roche reagents. Pooled samples from each treatment group were used for determination of lipoprotein subtypes by FPLC. ED50s (dose producing half-maximal effect) for the decrease in serum cholesterol and triglycerides were determined by nonlinear regression analysis.

In the LDLR null mouse dose-response study, LY2562175 caused a dose-dependent decrease in serum cholesterol and serum triglycerides. At the dose of 10 mg/kg, the decrease in cholesterol with LY2562175 was 80% below vehicle-treated animals, and the decrease in serum triglycerides was 76% from control group. The ED50 for serum cholesterol was determined to be 2 mg/kg and 3.4 mg/kg for serum triglycerides (**Table II, Figure 2**).

Table II. Total cholesterol- and triglyceride-lowering in LDL Receptor null mice following a 7 day dosing regimen*

Dose	Percent Change			
(mg/kg)	serum TG	total chol	VLDL	LDL
1	-25	-37	-59	-1
3	-46	-57	-78	-15
10	-76	-80	-96	-50
30	-83	-83	-98	-62
ED50	3.40 mg/kg	2.00 mg/kg		

* Male LDLR null mice were treated with varying doses of LY2562175 for 7 days. Total cholesterol and triglycerides were quantified by enzymatic analysis using a Hitachi 912 Clinical Chemistry Analyzer with Roche reagents, and lipoprotein fractions were quantified by FPLC.

The ZDF is an inbred rat model that through genetic mutation (fa mutation results in shortened leptin receptor protein that does not effectively interact with leptin) and a managed diet of Purina 5008 will closely mimic human adult onset diabetes (Type 2) and related complications. When fed a diet of Purina 5008, homozygous recessive males develop obesity, hyperlipidemia, fasting hyperglycemia and Type 2 diabetes. The obese female ZDF rat does not typically develop diabetes on standard rodent diets but does exhibit obesity, insulin resistance, and hyperlipidemia.

Treatment of female ZDF rats with LY2562175 results in a dose dependent lowering of plasma triglycerides in both the fasted and non-fasted states (**Table III**). When administered as a fixed dose combination with rosiglitazone, LY2562175 further lowers fasted and non-fasted plasma triglycerides. FPLC fractionation of the lipoproteins reveals that LY2562175-treatment results in a reduction in VLDL-C and a dramatic increase in HDL-C in this animal model. In fact the high dose of 30mpk resulted in a near doubling of HDL relative to vehicle controls. Thus, treatment of the female ZDF rat with FXR agonists causes dramatic reduction of circulating triglycerides and elevates HDL-cholesterol, and it is tempting to speculate that FXR agonists may be efficacious in treating components of diabetic dyslipidemia.

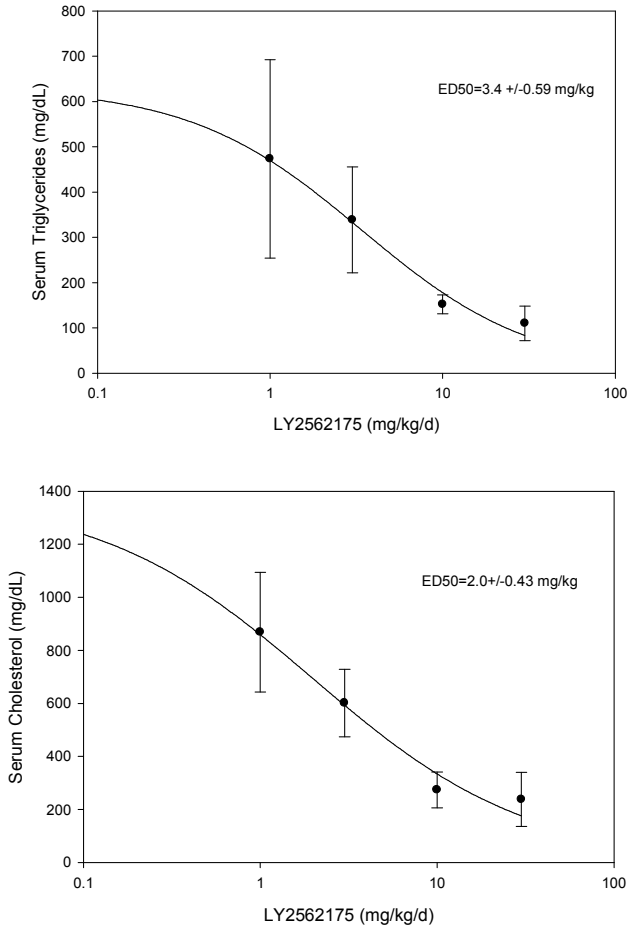


Figure 2. Male LDLR null mice were treated with varying doses of LY2562175 for 7 days. Total cholesterol and triglycerides were quantified by enzymatic analysis using a Hitachi 912 Clinical Chemistry Analyzer with Roche reagents (Mean ± SD; n=6). ED_{50}s (dose producing half-maximal effect) for the decrease in serum cholesterol and triglycerides were determined by nonlinear regression analysis.

Pharmacokinetics (PK) in Nonclinical Species. Pharmacokinetic studies were conducted in Sprague Dawley (SD) rats, Beagle dogs, and Cynomolgus monkeys to assess the oral and intravenous exposure for Compound 2562175. Several PK parameters, including the calculated oral bioavailability were also determined. IV doses were administered at 1 mg/kg (rat and dog) or 0.91 mpk (monkey) in a standard 5% Solutol / 5% EtOH in saline formulation. Oral/nasogastric doses were administered at 3 mpk in a standard vehicle (1.0 % CMC/ 0.25% PS80/ 0.05% antifoam in purified water). Samples were collected, processed to plasma, and analyzed by LC-MS/MS. The PK data are summarized in **Table IV**. Following IV administration, the mean terminal elimination half-life (T1/2) of LY2562175 ranged from approximately 2 to 10 hours, depending on the species. Clearance was low to moderate for all species where values were less than hepatic blood flow in each respective species.[8] Oral exposures were higher in non-rodent compared to rodent species. The Tmax ranged from approximately 1 to 3 hours for all species. The oral bioavailability for rats, dogs and monkeys was 21 ± 5%, 82%, and 24 ± 3%, respectively. The fraction unbound in rat and human plasma was 0.020 and 0.032 respectively.

Table III. Evaluation of LY2562175 alone or in combination with a low, fixed dose (0.3mpk) of rosiglitazone in female ZDF rats for 9 days*

Dose (mg/kg)	VLDL	LDL/lgHDL	HDL	Total
Vehicle	35	17.8	48.3	101.1
3	21.9	10.8	62.5	95.2
10	18.2	12.5	74.4	105.1
30	13.2	18.6	94.0	125.9
10 + Rosi	8.5	20.3	95.0	123.8
Rosiglitazone	26.3	19.0	61.3	106.5

*Female ZDF rats were treated with varying doses of LY2562175, rosiglitazone (rosi), or a fixed dose combination for 9 days. Lipoprotein fractions were quantified by FPLC and are expressed as mg/dl.

Pharmacokinetics in Human. A 2-cohort, 3-period, double-blind, placebo-controlled, single-dose escalation study was conducted at a single site, to determine the PK parameters of LY2562175. Two cohorts of 9 healthy subjects were included in this study. The cohorts were dosed alternatively in the course of a dose escalation with the following dose escalation scheme: 5 mg, 25 mg, 75mg, 200 mg, 400 mg, and 600 mg of LY2562175. After treatment with a single oral dose of LY2562175 in the dose range of 5 to 600 mg, the drug was well tolerated and maximum LY2562175 plasma concentrations were reached on average 2 to 3 hours post-dose (**Figure 3**). Concentration area under the curve and maximal concentrations reached had less than dose-proportional increases over the dose range studied. The mean T1/2 was similar across the dose range 75 to 600 mg LY2562175 and varied between 16.9 and 24.2 hours. The LY2562175 Cmax observed at the ED50 dose for lipid modulation in rodents was 4.62ng/mL (dashed line), and the human plasma concentration at the lowest dose tested (5mg) peaked at over twice that level and remained so to approximately 10 hours.

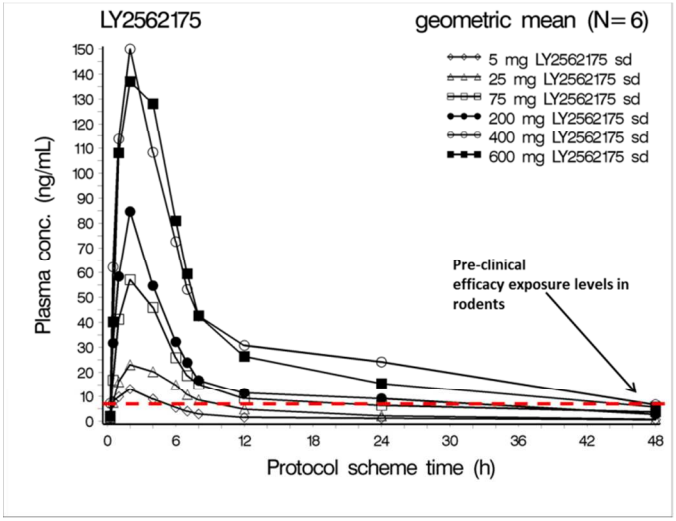


Figure 3. Human SAD Total Plasma Concentration vs Time Profiles for LY2562175.

Table IV: Summary of PK parameters after administration of LY2562175 to SD rats, Beagle dogs, or Cynomolgus monkeys. (Mean ± SD, N=2-3).

Species/Study	SD Rat		Beagle Dog		Cynomolgus Monkey	
Dose (Route)	**1 mpk (IV)**	**3 mpk (PO)**	**1 mpk (IV)**	**3 mpk (PO)**	**0.91 mpk (IV)**	**3 mpk (PO)**
$AUC_{0\text{-}24hr}$ (ng*h/mL)	577 ± 181	365 ± 178	1785 ± 90	4686	2302 ± 215	1813 ± 350
AUC Extrap (ng*h/mL)	586 ± 179	373 ± 177	1855 ± 124	4707	2322 ± 226	1831 ± 346
% AUC Extrap	2 ± 1	2 ± 1	4 ± 4	0.5	1 ± 1	1 ± 1
T1/2 (h)	2 ± 1	2 ± 0	10 ± 4	7	5 ± 1	9 ± 2
CL (mL/min/kg)	30 ± 8	---	9 ± 1	---	6 ± 1	---
Vdss (mL/kg)	1235 ± 622	---	2483 ± 832	---	597 ± 275	---
Co (ng/mL)	2527 ± 676	---	1184 ± 191	---	4222 ± 605	---
Tmax (h)	---	1.08 ± 0.88	---	0.75	---	3.33 ± 1.15
Cmax (ng/mL)	---	130 ± 96	---	1243	---	221 ± 66
F%	---	21 ± 5	---	82	---	24 ± 3

Conclusion. In summary, LY2562175 is a potent and partial agonist relative to GW4064 for human FXR as determined by cell-based transcriptional transactivation assays (Avg EC50=193 nM). Modulation of plasma lipids in LDLr KO mouse models reveals robust triglyceride and cholesterol lowering. Furthermore, LY2562175 reduces triglycerides significantly and elevates HDL-c by up to 95% in the insulin resistant female ZDF rat model. Due to the excellent lipid modulation profile in rodents as well as acceptable PK properties, LY2562175 was advanced into human clinical trial evaluation for dyslipidemia. Results from SAD studies revealed PK in humans that support once daily dosing.

ASSOCIATED CONTENT

Supporting Information. Experimental procedures and analytical data for Compound 1, in vitro and in vivo pharmacology procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written by M.J.G. and edited by L.F.M. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

FXR; farnesoid X receptor, NCS; n-chlorosuccinimide, SD; Sprague-Dawley, LDL; low density liproprotein, HDL; high density liproprotein, Rosi; rosiglitazone.

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