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Design, synthesis, and structure–activity relationship of podocarpic acid amides as liver X receptor agonists for potential treatment of atherosclerosis

Weiguo Liu,* Steve Chen, James Dropinski, Lawrence Colwell, Michael Robins, Michael Szymonifka, Nancy Hayes, Neelam Sharma, Karen MacNaul, Melba Hernandez, Charlotte Burton, Carl P. Sparrow, John G. Menke and Sheo B. Singh*

Merck Research Laboratories, P.O. Box 2000, Rahway, NJ 07065, USA

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Abstract—A series of podocarpic acid amides were identified as potent agonists for Liver X receptor α and β subtypes, which are members of a nuclear hormone receptor superfamily that are involved in the regulation of a variety of metabolic pathways including cholesterol metabolism. We recently reported podocarpic acid anhydride and imide dimers as potent LXR agonists. Through parallel organic synthesis, we rapidly identified a series of new podocarpate leads with stable structures exemplified by adamantyl- and phenylcyclohexylmethyl-podocarpic acid amides (**14** and **18**). Compound **18** exhibited LXRα/β 50/20 nM (binding affinity) and 33.7/35.3-fold receptor inductions. Synthesis, SAR, and biological activities of new podocarpate analogs are discussed.

It has been well-established in the medical community that higher level of cholesterol is a major risk factor associated with the occurrence of coronary heart disease and stroke. Pharmacologic intervention through efficient regulation of cholesterol biosynthesis, metabolism, acquisition, and transport in mammalian cells forms the scientific base for drug therapy of atherosclerosis. Liver X Receptors (LXR) are members of a superfamily of nuclear hormone receptors represented by two subtypes, LXRα and LXRβ.[1–3] Oxysterols have been identified as endogenous ligands for both subtypes.[4–7] These receptors have been shown to play significant roles in cholesterol homeostasis.[8] Upon ligand binding, LXRs form heterodimers with the retinoid X receptor (RXR), which recruit a variety of co-activators and activate the expression of a number of genes involved, directly or indirectly, in cholesterol and fatty acid metabolism including ABCA1, a cholesterol transporter protein. It has been shown that non-steroidal LXR agonists cause increased expression of ABCA1 and raise the HDL levels in mice. ABCA1 mediates the efflux of cholesterol out of the cells and onto the ApoA1 protein of HDL particles. Therefore, LXR agonists are expected to provide an opportunity for the development of drugs to increase reverse cholesterol transport and thus decrease the burden of atherosclerosis.[8]

In a previous recent account, we reported the identification of acetyl podocarpic acid anhydride (**1**) and imide dimer (**2**) as potent agonists of LXR α and β receptors.[9,10] Monomeric natural product, podocarpic acid (**3**), and various differently linked dimeric compounds were either significantly less active or inactive. While imide **2** exhibited good overall activity including in vivo activity, it lacked the required physical and pharmacokinetic (PK) properties to be considered for further development. During the SAR study of dimers, we observed that unsymmetrical dimers with stable linkages retained some LXR receptor-binding activity, albeit much weaker. It also became clear to us that all the active dimers were linked through at least one carboxyl functional group to the second molecule of the podocarpate. We reasoned that if we take one molecule of podocarpic acid and couple it through ester, amide, and imide bond with readily available alcohols and amines, we would generate a large number of podocarpic esters, amides, and imides. By changing the shape, size,

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* Corresponding authors. Tel.: +732 594 3222; fax:+ 732 594 6880; e-mail addresses: Weiguo_liu@merck.com; sheo_singh@merck.com

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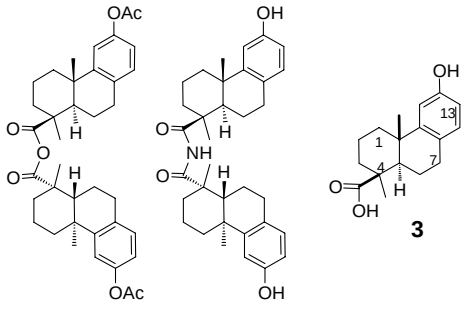
polarity, functionality, and stereochemistry of the amino donors, we would be able to fine-tune the SAR of these analogs toward obtaining potent LXR agonists. The discovery, synthesis, and SAR of selected esters (**4**–**7**), amides (**8**–**23**), imide (**24**–**25**), and C-linked diketone (**26**) are described.



Most of the primary and secondary amides presented in Table 1 were synthesized by the reaction of primary and secondary amines with unprotected podocarpic acid (**3**), either by activation of HOBt and EDAC in CH_2Cl_2 or Bop reagent in DMF in excellent yield. Synthesis of some of the hindered secondary amides and cyclic amides required protection of the phenolic group as a benzyl ether[10] and activation by either of the two reagents in DMF, followed by removal of benzyl ether by catalytic hydrogenolysis (Scheme 1). Esters were synthesized by acid chloride activation, followed by reaction with appropriate alkyl halides. Imides were synthesized by reacting benzyl protected podocarpic amide (**27**)[9,10] with appropriate acid chlorides (Scheme 2).

The diketone **26** was prepared in good yield by acylation of deprotonated podocarpic methyl ketone (**28**)[10] with adamantyl acid chloride, followed by hydrogenation (Scheme 3).

Deprotonation of the podocarpic amide (**18**) with sodium hydride, followed by reaction with diethyl phosphorochloridate, produced phosphonate (**29**), which upon metal ammonia reduction provided 13-deoxy podocarpic amide (**19**) in >70 overall yield (Scheme 4).

Like dimeric podocarpates, all compounds were first evaluated in LXRSPA α- and β-binding assay using {3-chloro-4-(3-(7-(2,3-ditritio-propyl)-3-trifluoromethyl-6-(4,5)-isoxazolyl) propylthio)-phenyl acetic acid, **30**, followed by measurement of in vitro agonist activity in a coactivator association assay using recombinant steroid receptor coactivator 1 (SRC1) protein and recombinant human LXRα and β ligand-binding domains in a homogeneous time-resolved fluorescence (HTRF) assay.[11] Cell-based transactivation (TA) assay was used to evaluate functional agonist or antagonist activities of compounds using a chimeric LXR construct in HEK-293 cells. This assay uses fusion proteins with the yeast Gal4 DNA-binding domain connected to the hinge region and the LBD domain of either LXR human receptors and has been previously described.[9,11] We prepared over 250 analogs of podocarpic acid predominated by amides together with a few esters, selected imides, and C-linked diketones. Polar amides (including O- and N-containing heterocycles) and esters with or without linkers were uniformly inactive. Representative examples of compounds are given in Table 1. The carboxy amide and the methyl ester did not exhibit any binding activity. Lipophilic amides and esters exhibited variable binding activity. Benzyl ester (**4**) exhibited binding activities with binding IC_{50} value of ~2.8 μM against both receptors. The allyl ester (**5**) was inactive, whereas the esters with four (e.g., **6**) and five (e.g., **7**) carbon chains showed modest activity. In the amide series, phenyl amide was completely inactive, whereas benzyl amide did not exhibit any binding affinity for α-receptor but exhibited binding affinity for β-receptor (IC_{50} = 3.9 μM). The extension of the chain length by one methylene group (e.g., phenethyl derivative, **8**) led to improvement of activities against both receptors (Table 1). However, *p*-hydroxylation of the phenethyl amide lost activity completely. Amides of amino acids with *tert*-butyl or *allyl* ester exhibited binding IC_{50} ranging from 1 to 5 μM but only rarely exhibited any activity in the functional HTRF assays. Amides with bulky alkyl groups with or without phenyl group exhibited some of the best activities. While a number of non-aromatic amides (e.g., **9**–**13**) exhibited sub μM to 5 μM IC_{50} values, adamantyl amides (**10**–**11**) exhibited better activities and were pursued further. The binding affinities of the primary (**10**) and the secondary (**11**) adamantyl amides were essentially identical, exhibiting IC_{50} values of ~0.10 and ~0.18 μM for α and β LXR receptors, respectively, displaying about 2-fold preference for the α-receptor. Shortening of the chain length (e.g., **14**) or movement of the nitrogen to the adjacent carbon (e.g., **15**) did not have any significant impact on the binding affinity. The ester substitution at C-9 of adamantyl ring (e.g., **16**) retained most of the binding affinity but hydrolysis of the ester to carboxylic acid lost all of the affinity (data not shown). Monohydroxy substitutions at C-5, C-7, or C-8 of the adamantyl ring resulted in inactive (IC_{50} > 10-50 μM) compounds. Likewise, reduction of the amide keto group of **10** to methylene lost the activity (α/β binding IC_{50} 7.04/8.50 μM). Substitution of podocarpic acid part of **10** with 13-hydroxy 13-iodo, 13-bromo, 7-keto retained only 5–10% of the activity (data not shown).

The binding affinity of these compounds correlated well with the coactivator association activity against the α-receptor but not against the β-receptor. The three best compounds of the series **10**, **11**, and **16** exhibited EC_{50} values of 0.23, 0.14, and 0.76 μM, respectively, against α-receptor in coactivator association HTRF assay and were full agonists. These compounds showed 22.6-, 29.5-, and 14.3-fold maximal induction, respectively, of the LXR α-receptor in the transactivation assay. Despite lack of stimulation against the β-receptor in the HTRF assay, these compounds exhibited 24.4-, 19.4-, and 19.7-fold TA induction, respectively, against the β-receptor. In contrast to dimeric series, the adamantyl imide (**24**) and C-linked diketone (**26**) derivatives had lost significant activities in all assays.

Table 1. Podocarpic acid ester, amides, imide, and C-linked diketone derivatives and their LXR activities

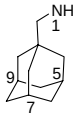
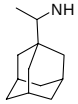
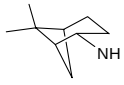
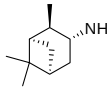
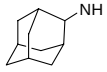
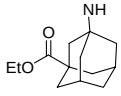
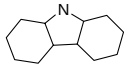
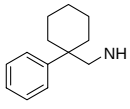
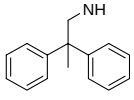
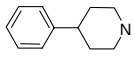
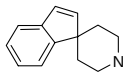
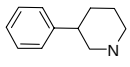
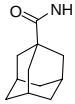
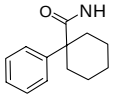
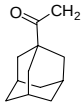
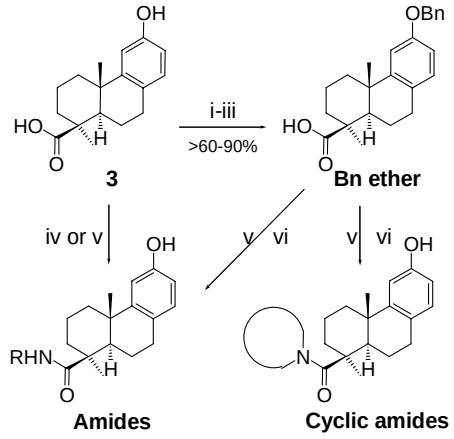
Compound	R-podocarpate	SPA binding (IC_{50}, μM)		Coactivator association HTRF assay (EC_{50}, μM)		Transactivation (EC_{50}, μM)		TA max fold induction	
		LXRα	LXRβ	LXRα	LXRβ	LXRα	LXRβ	LXRα	LXRβ
1	Acetyl anhydride dimer	0.002	0.002	0.002	0.002	0.010	0.010	60	54
2	Imide dimer	0.001	0.001	0.001	0.001	<0.003	<0.003	24	30
4	Ph CH_2O	2.8	2.9	>15	>15	NA	NA	NA	NA
5	$CH_2{=}CHCH_2O$	>15	>15	>50	>50	NT	NT	NT	NT
6	$CH_2{=}CHCH_2CH_2O$	5.06	3.85	>50	>50	NT	NT	NT	NT
7	$CH_2{=}CHCH_2CH_2CH_2O$	3.8	5.0	>50	>50	NT	NT	NT	NT
8	$PhCH_2CH_2NH$	2.93	2.22	5.2	>50	40% at 10[a]	28% at 10[a]	10.5	11.5
9		3.1	4.12	3.1	>50	NA	NA	2.2	2.8
10		0.10	0.19	0.23	>50	0.86	50% at 10[a]	22.6	24.4
11		0.09	0.18	0.14	>50	136% at 10[a]	49% at 10[a]	29.5	19.4
12		0.35	0.31	0.24 (40%)[b]	>50	63% at 10[a]	13% at 10[a]	11.7	7.1
13		0.27	0.20	0.28	>50	1.70	78% at 10[a]	12.7	23.3
14		0.14	0.17	0.22	0.22 (27%)[b]	130% at 3[a]	81% at 10[a]	41.2	26.8
15		0.13	0.10	0.32	>50	0.90	3.80	2.6	2.3
16		0.03	0.53	0.76	>10	0.83	51% at 3[a]	14.3	19.7
17		0.30	0.19	0.32	>50	430% at 10[a]	139% at 10[a]	47.5	44.5
18		0.05	0.02	0.05	>50	164% at 3[a]	90% at 3[a]	33.7	35.3
19	12-deoxy-**18**	0.20	0.13	0.17 (49%)	>50	98% at 10[a]	40% at 10[a]	18.9	15
20		0.49	0.42	0.36	>50	42% at 10[a]	21% at 10[a]	21.7	5.5
21		0.20	0.18	0.07	0.07 (31%)[b]	92% at 1[a]	95% at 1[a]	22.8	22.1
22		0.75	0.50	0.43	1.5	175% at 3[a]	275% at 1[a]	5.6	4.4
23		0.36	0.37	0.15	0.1 (25%)[b]	144% at 3[a]	111% at 3[a]	24.2	32.1

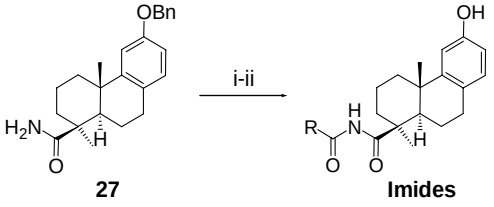
Table 1 (*continued*)

Compound	R-podocarpate	SPA binding (IC_{50}, μM)		Coactivator association HTRF assay (EC_{50}, μM)		Transactivation (EC_{50}, μM)		TA max fold induction	
		LXRα	LXRβ	LXRα	LXRβ	LXRα	LXRβ	LXRα	LXRβ
24		0.29	0.29	1.5 (38%)[b]	>50	20% at 10[a]	NA	5.1	1.7
25		0.42	0.12	0.17	>50	NA	NA	NA	NA
26		1.67	1.01	>50	>50	62% at 10[a]	13% at 10[a]	5.4	4.4
30	Control compound	0.035	0.025	0.035	0.016	1.79	1.08	20	35

[a] The % activation did not plateau.
[b] Partial agonist; NT (not tested); NA (not active at 10 μM); **30** {3-chloro-4-(3-(7-(2,3-ditritio-propyl)-3-trifluoromethyl-6-(4,5)-isoxazolyl) propyl-thio)-phenyl acetic acid)}.



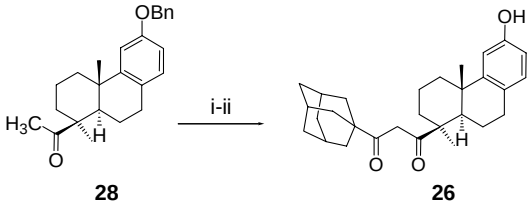
Scheme 1. Reagents: (i) $TMSCHN_2$, MeOH, PhH; (ii) Cs_2CO_3, BnBr, DMF; (iii) K-*t*-BuO, DMSO, H_3O^+; (iv) HOBt, EDAC, CH_2Cl_2, primary amines (v) Bop reagent, DMF, primary or secondary or cyclic amines; (vi) H_2, 10% Pd/C, 45 psi.



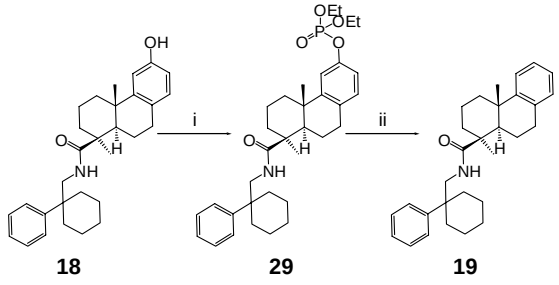
Scheme 2. Reagents: (i) NaHMDS, THF, acid chlorides, (ii) H_2, Pd/C, 45 psi.

One of the other bulky aliphatic cyclic amides that is worth mentioning is **17** that exhibited α/β binding IC_{50} of 0.30/0.19 μM, coactivator association EC_{50} of 0.32/ > 50 μM, and α/β TA fold induction of 47.5/44.5.

The phenethyl lead was explored further by coupling of commercially available aromatic amines with various



Scheme 3. Reagents: (i) NaHMDS, THF, Adamantyl chloride; (ii) H_2, Pd/C, 45 psi.



Scheme 4. Reagents: (i) NaH, THF, diethyl phosphorochloridate; (ii) diethyl ether, liquid NH_3, Li, −78 °C.

design elements, such as variation of the length of the linker and introduction of constraints leading to the synthesis of primary, secondary, and cyclic amides. The SAR of this class of compounds was very complex and clustered in three groups. The most interesting compounds of the group are presented in Table 1. Of these, the primary amide **18** and the cyclic amide **21** were most potent and exhibited α/β binding IC_{50} values of 0.05/0.02 and 0.20/0.18 μM, respectively. While the primary aromatic amide **18** exhibited potent activity (EC_{50} 0.05 μM) in the coactivator association assay against the α-receptor, it was completely inactive against the β-receptor. In contrast, the cyclic aromatic amide **21** was a full agonist (EC_{50} 0.07 μM) against the α-receptor

and a partial agonist (EC_{50} 0.07 μM) against the β-receptor (maximal stimulation observed was only 31% for the β-receptor). The primary and secondary amides (e.g., **18** and analogs, **20**) were consistently devoid of the β-receptor HTRF activity and exhibited selectivity for α-receptor, while all cyclic amides (e.g., **21**–**23**) exhibited β-receptor HTRF activity and did not show any selectivity. The amide **18** was a potent activator and exhibited 33.7-fold activation of the α-receptor and 35.3-fold activation of the β-receptor. Cyclic amide **21** was also a potent activator, providing 92% and 95% max activation of α and β receptors in TA assays at 1 μM affording 22.8- and 22.1-fold inductions, respectively.

Of these two most active leads, amide **18** was selected for further SAR studies. First, N-methylation caused 10- to 15-fold loss of activities. Replacement of the cyclohexyl ring with cyclopropyl, cyclobutyl, cyclopentyl rings and dimethyl group, substitution in the aromatic ring with *m*- and *p*-OMe, *m*- and *p*-CO_2Me uniformly resulted in the reduction of binding affinity by 2- to 20-fold but substitution with *m*- or *p*-CO_2H lost affinity completely. The elimination of the phenolic group (e.g., **19**) and the conversion to imide analog (**25**) resulted in a reduction of binding affinity and agonist activities.

Movement of the nitrogen one carbon (e.g., **23**) or constraining the flexibility (e.g., **22**) of the cyclic amides led to the loss of activity, indicating that both position of the nitrogen and slight flexibility are important for binding affinities and agonist properties. Derivatization of the phenolic group as ether or ester (except acetate) was detrimental for the activity in all series.

Before in vivo studies, Male SD rats PK studies of **14** and **18** were conducted. Rats were dosed at 1 mg/kg intravenous ($N = 2$) in EtOH:PEG400:H_2O (20:30:50 v/v/v, 1 mg/mL) and 2 mg/kg oral ($N = 3$) by gavage. The compound was recovered from blood by acetonitrile precipitation and analyzed by LC-MS/MS with external standard. Amide **14** afforded iv AUC of 1.03 μM.h.kg/mg, Clp 42 mL/min/kg, Vdss of 2.67 L/kg and $t_{1/2}$ of 1.15 h; and po AUC 0.01 μM.h.kg/mg, C_{max} 0.01 μM and F 1.1%. Amide **18** gave iv AUC of 0.47 μM.h.kg/mg, high Clp 82 mL/min/kg, high Vdss of 9.5 L/kg and $t_{1/2}$ of 1.8 h; and po AUC 0.04 μM.h.kg/mg, C_{max} 0.03 μM and F 13.5%. The amide **18** was evaluated for in vivo efficacy in C57Bl/6J male mice (fed Chow diet) at 30 mg/kg twice daily by oral administration for 7 days. Cholesterol and triglyceride levels were measured. Unlike imide **2**, this compound did not show any effect on the lipid levels potentially due to its lower (~50-fold) intrinsic potency (compared to **2**) and lower exposure level.

In summary, in this paper we have described podocarpic amides as potent agonists of LXR, defined broad SAR, and extended our initial studies of dimers.

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