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Analogues of 2-phenyl-ethenesulfonic acid phenyl ester have dual functions of inhibiting expression of inducible nitric oxide synthase and activating peroxisome proliferator-activated receptor γ

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ABSTRACT

We identified a series of 2-phenyl-ethenesulfonic acid phenyl ester analogues as novel dual-function agents that suppressed nitric oxide production in lipopolysaccharide/interferon γ-stimulated RAW264.7 cells and activated peroxisome proliferator-activated receptor γ (PPARγ) in a cell-based transactivation assay. Western blot analysis demonstrated that these compounds inhibit the expression of inducible nitric oxide synthase protein, and scintillation proximity assay validated their ability to bind to PPARγ. Our studies provide the basis for developing these dual-function agents for anti-inflammation and anti-atherosclerosis therapy.

Macrophages could be an emerging therapeutic target in atherosclerosis.[1] Inducible nitric oxide synthase (iNOS) contributes to the size of atherosclerotic lesions in apoE-deficient mice and thus the inflammatory process of plaque development; indeed, downregulated iNOS or nitric oxide (NO) production reduced evidence of atherosclerosis in such mice.[2,3] Moreover, iNOS is induced in activated macrophages to produce a large quantity of NO, whereas agonists of peroxisome proliferator-activated receptor γ (PPARγ) negatively regulate the macrophage activation and the accompanying iNOS expression and NO production.[4–5] iNOS is a pro-inflammatory factor, and inhibition of its expression results in anti-inflammatory effects in vitro and in vivo.[6–8] Activation of PPARs, including PPARγ plays an important role in attenuating atherosclerosis and inflammation.[9–13] Thus, iNOS and PPARs play opposite roles in the inflammatory response and the progression of atherosclerosis.

Intensive effort has been invested in the development of drugs involving PPARγ agonists as therapeutic agents[11,14], although naturally occurring compounds such as fatty acids, eicosanoids, and 15-deoxy-△12,14-prostaglandin J2 (15d-PGJ2) also bind to and activate PPARγ[15,16] 15d-PGJ2 is synthesized through prostaglandin D2 and potently inhibits iNOS expression in macrophages. However, other highly specific PPARγ agonists—BRL49653 (rosiglitazone), troglitazone, and GW2090—have much less inhibitory activities for iNOS than does the natural ligand 15d-PGJ2.[4] In RAW264.7 cells, 15d-PGJ2 and BRL49653 were proposed to activate PPARγ, respectively, by direct interaction with the N-terminal domain of the cAMP response-element binding (CREB) protein and thus competes for limited amounts of the general coactivators CREB protein and p300, thereby transrepressing iNOS expression.[17] Additionally, further studies indicated that ligand-dependent SUMOylated PPARγ targets to nuclear receptor corepressor on the iNOS promoter in LPS-stimulated macrophages whereby NF-κB signaling pathway is activated. This in turn resulted in interferes with clearance of the corepressor complex containing nuclear receptor corepressor and histone deacetylase 3, and thus maintain the iNOS promoter in the repressed state with presence of activated NF-κB.[18–19]

RAW264.7 cells, a murine monocyte-like cell line, upon stimulation with LPS/IFNγ are activated to become macrophages and induced to express pro-inflammatory factors, for example, iNOS and COX-II. iNOS synthesizes a large quantity of NO in these cells.[7] 15d-PGJ2 and BRL49653 inhibit NO production, with IC_{50} values of 2.2 μM[7] and 17.5 μM (our unpublished data), respectively, and both bind to and activates PPARγ but not PPARδ in these cells (data not shown). Thus, compounds with properties similar to 15d-PGJ2 or BRL49653 could be a unique class of therapeutic agents with potential for treating atherosclerosis. This exploratory study aimed to find agents with the dual functions of inhibiting NO production and activating PPARγ for a novel class of agents with not only

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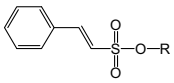
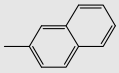
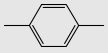
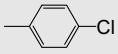
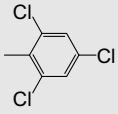
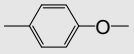
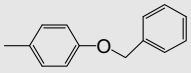
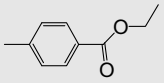
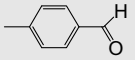
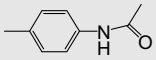
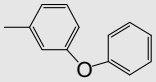
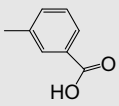
anti-inflammatory effects but also effects to reduce the progression of atherosclerosis.

Two projects involving high throughput screening that shared an overlapping set of 10,000 compounds revealed three compounds with the dual functions of suppressing NO production in LPS/IFNγ-stimulated RAW264.7 cells[6] and of binding affinity to PPARγ on scintillation proximity assay of competition binding with ^{3}H-BRL49653.[20,21] These compounds were 2-phenyl-ethenesulfonic acid phenyl ester (**1a**), 2-phenyl-ethenesulfonic acid 4-chlorophenyl ester (**1e**), and 2-methyl-4-nitro-quinoline 1-oxide. Initially, 4-nitro-quinoline 1-oxide, a commercially available analogue of 2-methyl-4-nitro-quinoline 1-oxide, was tested at 10 μM for NO suppression but revealed no significant activity (data not shown). On the other hand, we collected and assayed several analogues of **1a** and **1e** for NO suppression in terms of IC_{50} values. We also assessed their PPARγ transactivation activity (%) relative to that of 15d-PGJ2 by use of a UAS_3-luciferase reporter system and a construct of a Gal4-DNA binding domain fused with PPARγ ligand binding domain.[22,23] Some of these compounds showed potent dual functions. Therefore, we collected additional related analogues[24] for further investigation and analysis of structure–activity relations.

Substitutes of the phenyl group of the sulfonic acid phenyl ester side resulted in a differential degree of influence in the dual functions of NO suppression and PPARγ activation (Table 1). Compound **1a** has an IC_{50} value of 6.66 μM for NO suppression and 33.4%

Table 1
Dual functions and activities of 2-phenyl-ethenesulfonic acid phenyl ester compounds



Compound		NO suppression IC_{50}[a] (μM)	Cytotoxicity GI_{50} (μM)	PPARγ binding competition IC_{50}[a] (nM)	PPARγ transactivation[a] (%)
1a		6.66 ± 3.24	>80	1326 ± 373	33.4 ± 7.8
1b		6.18 ± 3.42	>80	1088 ± 208	25.8 ± 1.8
1c		2.77 ± 0.47	>80	407 ± 10	31.3 ± 4.0
1d		3.45 ± 0.79	62.5	1187 ± 149	33.2 ± 11.4
1e		2.35 ± 0.33	75.3	302 ± 54	24.5 ± 4.1
1f		1.83 ± 0.22	>80	1439 ± 183	60.9 ± 9.3
1g		7.45 ± 0.47	ND[b]	1294 ± 484	22.7 ± 6.4
1h		9.30 ± 1.30	>80	703 ± 130	16.0 ± 9.5
1i		9.88 ± 1.95	>80	747 ± 32	19.1 ± 4.3
1j		9.72 ± 1.77	>80	2098 ± 611	13.8 ± 1.5
1k		19.93 ± 1.46	>80	10712 ± 1223	8.4 ± 2.6
1l		16.20 ± 0.81	>80	382 ± 122	41.6 ± 9.5
1m		21.63 ± 3.55	>80	13521 ± 901	19.7 ± 3.6

The IC_{50} for NO suppression and cytotoxicity of the test compounds were measured in RAW264.7 cells stimulated with LPS/IFNγ and PPARγ transactivation activity in CV1 cells transfected with the plasmids of chimeric receptor pSG424-PPAR γ ligand binding domain and UAS3 luciferase reporter gene; scintillation proximity assay was used for analysis of PPARγ binding competition IC_{50}.

[a] Data are means ± SD.

[b] ND, not determined.

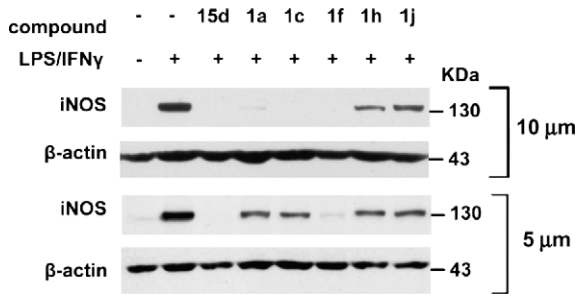


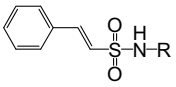
Figure 1. Western blot analysis of iNOS protein expressed in RAW264.7 cells stimulated with LPS/IFNγ and treated with 2-phenyl-ethenesulfonic acid phenyl ester compounds or 15d-PGJ2 (15d) at the concentrations indicated. β-actin was an internal loading control.

PPARγ transactivation activity relative to 15d-PGJ2. Replacing the phenyl group (**1a**) with naphthalene (**1b**) did not significantly affect the compound's functions, whereas introducing a methyl group at the *para* or *meta* positions of the phenyl group (**1c, 1d**, respectively) increased NO suppression activity 2.4- and 1.9-fold, respectively. Moreover, introducing a chloride at the *para* position or three chlorides at positions 2, 4, or 6 of the phenyl group (**1e, 1f**, respectively) increased NO suppression activity by 2.8- and 3.6-fold, respectively. Other substitutions at the *para* position decreased NO suppression activity, by 0.12-fold for a methoxyl group (**1g**), 0.40-fold for a methoxyl-benzene group (**1h**), 0.46-fold for an acid ethyl ester group (**1i**), 0.46-fold for an aldehyde (**1j**), and 1.99-fold for an *N*-acetamide group (**1k**). Substitutions at the *meta* position with a phenoxyl group (**1l**) decreased the NO suppression activity by 1.43-fold, whereas substitution with an acid (**1m**) further decreased the suppression activity 2.25-fold.

The results for PPARγ activation were similar to those for NO suppression (Table 1). For instance, compound **1f** exerted the most potent activities for both NO suppression and PPARγ activation and compounds **1k** and **1m** the least. Compounds with the worst competition for binding to PPARγ exhibited low PPARγ activation, and those with better competition did not achieve better PPARγ activation, as was expected. In addition, western blot results (Fig. 1) examining the compounds' effects on protein expression of iNOS were related to those for their activity for NO suppression (Table 1).

For extended search, we first used the function for NO suppression to examine other kinds of analogues of 2-phenyl-ethenesulfonic acid phenyl ester: 2-phenyl-ethenesulfonic acid phenylamides (**2a** to **2i**), 2-phenyl-ethanesulfonic acid phenyl esters (**3a** to **3g**), and phenyl-methanesulfonic acid phenyl esters (**3h** to **3j**). However, these compounds, at 10 μM, with substitutes at the phenyl group of sulfonic acid phenyl ester side, exerted only slight or non-significant activity for NO suppression (Tables 2 & 3). Moreover, in contrast to compound **1f**, its counterpart, ethanesulfonic compound **3c**, did not exhibit any PPARγ transactivation activity (data not shown). Furthermore, compounds **1a** to **1m**, as well as 15d-PGJ2 and BRL49653, exerted no detectable PPARδ TA activities

Table 2
NO suppression activity of 2-phenyl-ethenesulfonic acid phenylamide compounds (10 μM) in RAW264.7 cells stimulated with LPS/IFNγ



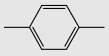
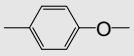
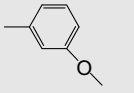
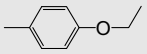
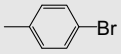
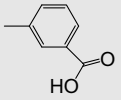
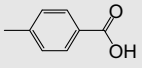
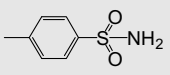
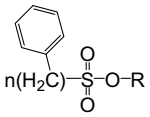
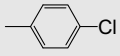
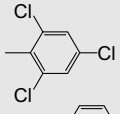
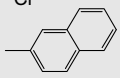
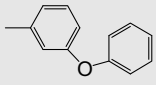
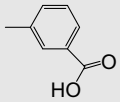
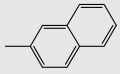
Compound		NO suppression activity (%)
2a		4
2b		8
2c		5
2d		12
2e		0
2f		5
2g		1
2h		2
2i		0

Table 3
NO suppression activity of 2-phenyl-ethanesulfonic acid phenyl esters (10 μM) and phenyl-methanesulfonic acid phenyl esters (10 μM) in RAW264.7 cells stimulated with LPS/IFNγ



Compound		*n* = 1 or 2	NO suppression activity (%)
3a		2	2
3b		2	4
3c		2	10
3d		2	6
3e		2	0
3f		2	11
3g		2	6
3h		1	3
3i		1	7
3j		1	0

(data not shown). Thus, ethenesulfonic acid is an important pharmacophore for NO suppression and for the dual functions of 2-phenyl-ethenesulfonic acid phenyl ester and its analogues with substitutes at the phenyl group of sulfonic acid phenyl ester side.

In summary, we have identified a series of 2-phenyl-ethenesulfonic acid phenyl ester analogues as novel agents with dual functions: the agents inhibited the expression of iNOS in LPS/IFNγ-stimulated RAW264.7 cells and activated the PPARγ isoform in a cell-based transactivation assay. These analogues warrant further development for anti-inflammation and anti-atherosclerosis therapy. Their detail mechanisms of molecular action in competing common transcriptional coactivators[17] or targeting to nuclear receptor corepressors[18] to repress iNOS expression upon binding to PPARγ ligand binding domain are needed to be determined.

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20. Scintillation Proximity Assay (SPA)-SPA allows the rapid and sensitive assay of a wide variety of molecular interactions in a homogeneous system. Microscopic beads (Amersham Pharmacia Biotech) containing scintillant that can be stimulated to emit light were used in SPA. This stimulation event only occurs when radiolabelled molecules of interest, in our case ^{3}H-BRL49653, are bound to the surface of the bead. Competition with the reference radiolabelled ^{3}H-BRL49653 binding to PPARγ involved adding test compounds individually to the SPA in the presence of ^{3}H-BRL49653 as described (Ref. 21). The IC_{50} was calculated from at least 8–10 concentrations of each individual compound used in the competition SPA. The SPA reaction buffer consists of 10 mM Tris–Cl, pH 7.2, 1 mM EDTA, 10% (W/V) glycerol, 10 mM Sodium molybdate, 1 mM DTT, 0.5 mM PMSF, 2 mg/ml benzamidine, 0.1% dry milk powder, 10 nM ^{3}H-BRL49653 (American Radiolabeled Chemicals), 5 nM GST-PPARγ (LBD) recombinant protein, diluted 200× goat anti-GST Ab (Amersham Pharmacia Biotech), protein A-yttrium silicate SPA beads (Amersham Pharmacia Biotech), and appropriate concentrations of test compounds. The reaction was carried out at 10 °C for overnight with mild shaking and the intensity of scintillation was detected with use of TopCount·NXT™ Microplate Scinetillation and Luminescence Counter (Packard).
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22. PPARγ Transactivation Assay-Human PPARγ full-length cDNA was cloned from a human brain library (cerebellum Marathon-ready cDNA library, Clontech) into pENTR-TOPO vector (Invitrogen). Subsequently, PPARγ LBD (residues 199–477) was fused with Gal4 DBD domain (residues 1–147) to generate a chimeric construct Gal4DBD-PPARγLBD in a pSG424 fusion vector with GAL 1–147. The CV1 cells, seeded 2.5×10^{4} cells per well in 24-well plates, were transfected with the plasmids of chimeric receptor pSG424-PPARγLBD and UAS3 luciferase reporter gene (Ref. 23) (a gift of Gal4-reporter from Dr. R.M. Evans, San Diego, CA), as well as pCMV-βGal using FuGene[6] (Roche) overnight. Before compound treatment, the transfectant cells were washed with culture medium twice. The compound effects were examined after 18- to 24-h treatment. Luciferase and β-galactosidase assays involved use of a Steady-Glo luciferase assay system (Promega and Galacto-Star (Tropix)) according to the manufacturer's instructions. Luminescence was measured in a TopCount·NXT™ Microplate Scinetillation and Luminescence Counter (Packard). The luciferase activity was normalized with β-galactosidase activity. The PPARγ transactivation-activity activated by the treatment of 10 μM 15d-PGJ2 was used at 100% to calculate the other assay activity with each individual compound treatment.
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24. RAW264.7 cell culture, Griess assay, MTS assay for cytotoxicity (GI_{50}), and chemicals, as well as MTS, were as described (Ref. 6). Nitrate was reduced to nitrite with nitrate reductase and determined spectrophotometrically with Griess reagent at OD_{540}. The assayed compounds were purchased from ChemDiv Inc. (San Diego, CA) and FAB-Mass and ^{1}H NMR as spectra data of these compounds were examined for purity. A part of compounds were also verified on liquid chromatography/mass spectrometry (Agilent Technologies) at NHRI.