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Discovery of a Novel 2,6-Difunctionalized 2*H*-Benzopyran Inhibitors Toward Sphingosylphosphorylcholine Synthetic Pathway as New Anti-inflammatory Target

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Novel 2,6-difuctionalized 2*H*-benzopyrans were synthesized and evaluated for a sphingosylphosphorylcholine (SPC) inhibitor. The synthetic 2*H*-benzopyrans **1c** and **3a** showed high potency in SPC-induced cell proliferation assay (IC_{50} < 20 nM). Neither hERG K^+ channel binding (> 10 μM) nor CYP inhibitions (> 10 μM) were observed. Also, the simple structure-activity relationship (SAR) results were obtained from analysis of 2*H*-benzopyran derivatives **1-3** and the anti-SPC effect of 2*H*-benzopyran **1c** was confirmed by a HUVEC tube formation assay.

Key Words : Sphingosylphosphorylcholine, Anti-inflammatory inhibitors, Drug-like library, 2*H*-benzopyran, Small molecules

Introduction

Sphingolipids are ubiquitous in mammalian membranes. Usually, sphingomyelin (SM) is metabolized to ceramide by sphingomyelinase (SMase). The activated SMase leads to induction of apoptosis and oxidative stress.[1] Meanwhile SM can be metabolized into a lyso-form, sphingosylphosphorylcholine (SPC, Fig. 1) instead of ceramide by SM deacylase.[2] SM deacylase (EC 3.5.1.109) from epidermis of atopic dermatitis (AD) patients has been characterized using [1-^{14}C] palmitoylsphingosine as a substrate. Unusual high activity of SM deacylase in the skin of AD would compete with SMase to occupy their common substrate-SM, which consequently results decreased level of ceramide and diminishes skin barrier compared with healthy controls. SM deacylase was characterized as a protein of 40 kDa with pI of 4.2. However, the putative amino acid sequence and the gene coding human SM deacylase were not elucidated.

SPC has been known to be naturally present in plasma and extensive studies have shown the diverse and complex effects of SPC.[3] Many dermatological investigations have shown that SPC increased the production of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) or interleukin-6 (IL-6) and promoted wound healing through the secretion of connective tissue growth factor and adhesion molecules.[4] Generally, SPC has been proposed as a deleterious factor in skin pathology[5] and is known to elicit intracellular calcium, reactive oxygen species (ROS) and cell migration in endothelial cells.[6] On the other hand, in the vascular system the effect of SPC is rather regarded as beneficial and accepted as an athero-protective factor. Murch *et al.* showed that lysophosphatidylcholine (LPC) and SPC reduced organ injury and dysfunction in rodent models.[7]

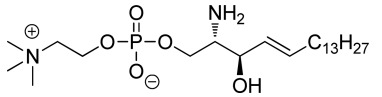


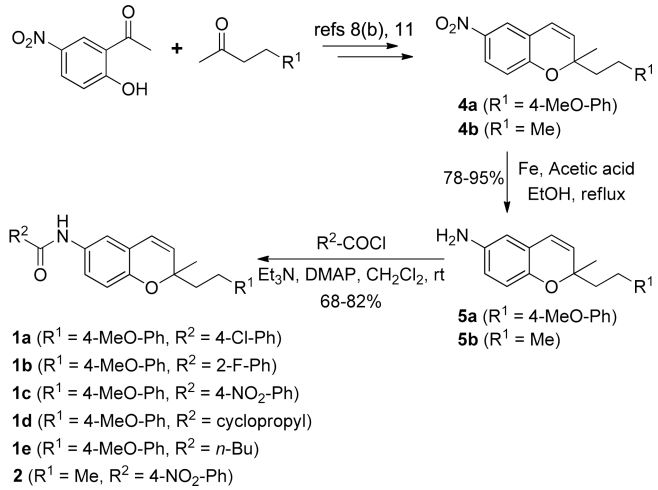
Figure 1. Structure of sphingosylphosphorylcholine (SPC).

Thus, the modulation of SPC action helps to control inflammation and pruritus. As part of an ongoing drug discovery project, we needed to discover a novel drug-like small molecule of SPC inhibitors. Herein, we present the discovery of novel potent SPC inhibitors by SPC-induced proliferation assay and cell-based anti-SPC assay with HUVEC tube formation.

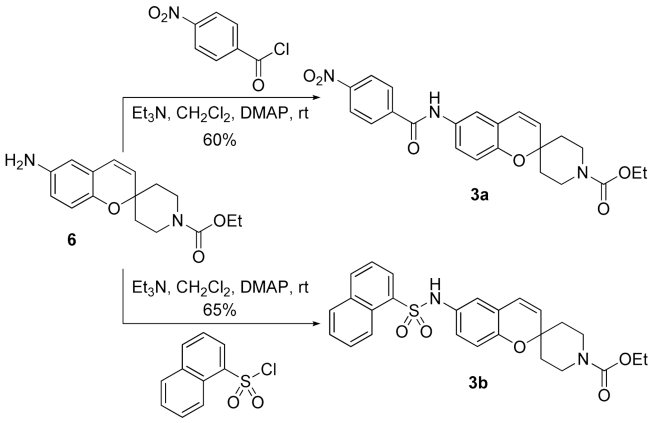
Results and Discussion

Chemistry. Initially, our in-house chemical library, which consists of diverse small molecules,[8] has been subjected to an SPC-induced proliferation assay. Among these molecules, we directed our interest to 2*H*-benzopyran derivatives because of a well-known privileged scaffold showing a variety of biological activites.[9] The 2*H*-benzopyran derivatives displayed high SPC inhibitory effect (> 75%) at 0.25 μM. The syntheses of 2,6-difunctionalized 2*H*-benzopyran derivatives **1-3** are depicted in Schemes 1 and 2.

The synthesis of 2*H*-benzopyrans **1** has been introduced from the known 2-(4-methyoxyphenethyl)-2-methyl-6-nitro-2*H*-chromene (**4a**),[10] which was prepared by cyclization of 6-hydroxy3-nitroacetophenone with 4-(4-methoxyphenyl)-



Scheme 1. Synthesis of 2*H*-benzopyrans **1** and **2**.



Scheme 2. Synthesis of 2*H*-benzopyrans **3**.

butan-2-one, reduction of ketone, mesylation, and elimination of mesylate.[11] 6-Amino-2*H*-benzopyran **5a** was obtained by the reduction of the nitro group in **4a**. The acylation with appropriate acid chlorides of amine **5a** provided the 2*H*-benzopyran derivatives **1** in high overall yields. Also, 2*H*-benzopyran **2** was obtained with a similar method for **1** from 6-nitro-2*H*-benzopyran **4b** (Fig. 2) (Scheme 1).

The preparation of spiro-benzopyran derivatives **3** is outlined in Scheme 2. The known *N*-[ethylcarbamate-spiro(2*H*-1-benzopyran-2,4-piperidine)-6-yl]amine **6**[8a] was converted to acyl spiro-benzopyran **3a** and sulfonyl spiro-benzopyran **3b** with 4-nitrobenzoyl chloride and naphthalene-1-sulfonyl chloride, respectively (Scheme 2).

Biology. The synthesized 2*H*-benzopyrans **1**-**3** (Fig. 2) were subjected to screening for SPC inhibitory effect and obtained IC_{50} values (Table 1). The screening was carried out in a 96-well format in an NIH3T3 cell line with [^{3}H]-thymidine proliferation.

The simple structure-activity relationship (SAR) analysis showed the effect of 6-amino substituent (R^2 in **1** and **2**; acyl or sulfonyl in **3**) and C-2 substituents (R^1 in **1** and **2**; and spiro part in **3**) on the 2*H*-benzopyran skeleton. When C-2 substituents are methyl and 4-methoxyphenethyl (R^1 = 4-OMe-Ph; **1a**-**1e**), 4-nitrobenzoyl derivative **1c** exhibits high SPC inhibitory activity (IC_{50} = 9 nM) and the *n*-butyl (**1e**) moiety is more active than the cyclopropyl (**1d**) moiety in

Table 1. SPC-induced proliferation and MTT assay

Entry	Product	Yields (%)[a]	%inhibition at 0.25 μM[b]	IC_{50} (nM)[c]	MTT % viability at 2.5 μM[d]
1	**1a**	82	96.4	236	64.7
2	**1b**	81	82.8	123	62.5
3	**1c**	78	91.1	9	91.4
4	**1d**	70	81.3	607	67.3
5	**1e**	68	91.0	173	77.0
6	**2**	73	78.5	47	77.5
7	**3a**	60	78.0	19	68.5
8	**3b**	65	95.4	30	71.6

[a]Isolated yields from **5** or **6**. [b,c][^{3}H]-thymidine SPC proliferation assay in NIH3T3 cell line and all data were obtained from triplicated experiments. [d]Cytotoxicity was determined in parallel with SPC proliferation assay by MTT methods

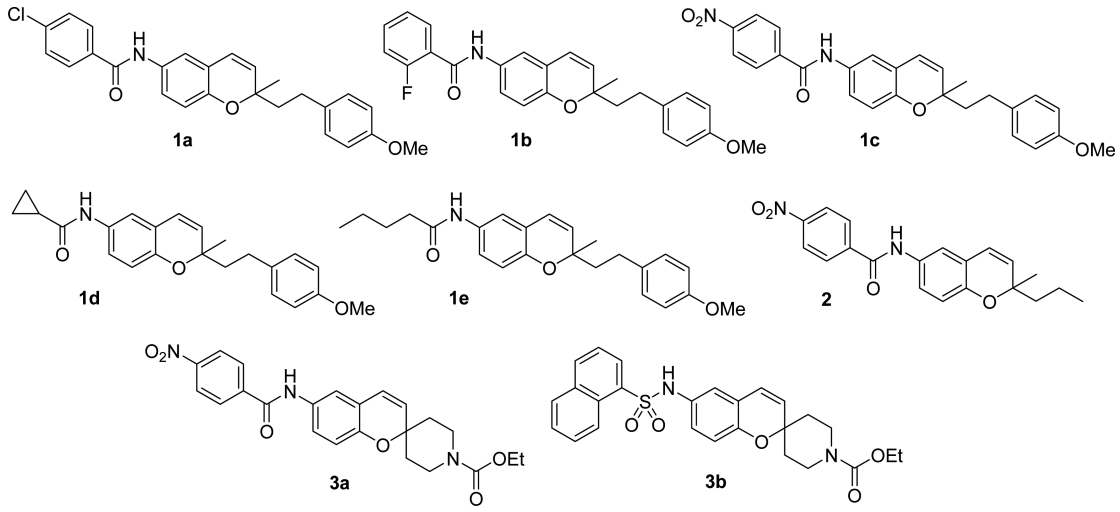


Figure 2. Benzopyran derivatives with SPC inhibitory effect.

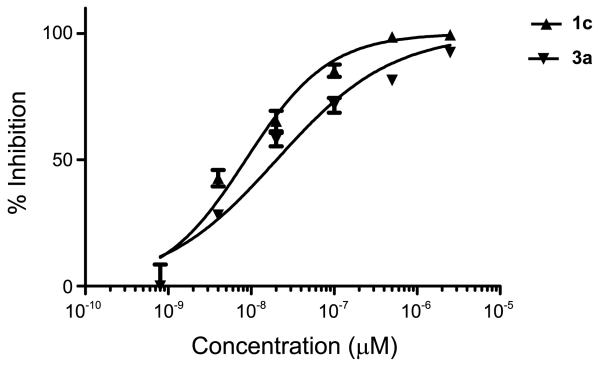


Figure 3. Suppression of the SPC-induced proliferation by compounds **1c** and **3a**.

Table 2. CYP450 inhibition and hERG assay of **1c** and **3a**

Product	IC_{50} of CYPs inhibition (μM)[a]	IC_{50} of hERG binding (μM)[b]
1c	1A2 > 10 2C9 > 10 2C19 > 10 2D6 > 10 3A4 > 10	> 10
3a	1A2 > 10 2C9 > 10 2C19 > 10 2D6 > 10 3A4 > 10	> 10

[a]CYP inhibition was evaluated using CYP membrane prep and luciferin-tagged substrates. [b]hERG binding was measured by [^{3}H]-astemizole binding onto hERG K^+ channel/HEK293 membranes.

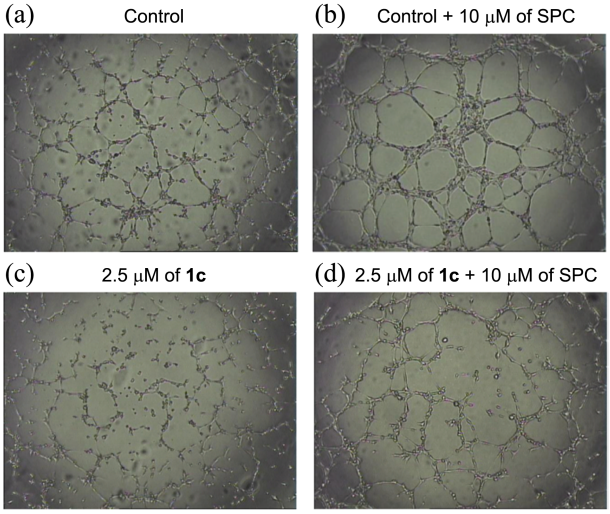


Figure 4. HUVEC tube formation assay. Representative photographs of HUVEC tube formation induced by control (vehicle, 0.1% DMSO) (a), 10 μM of SPC (b), 2 μM of compound **1c** (c), or 10 μM of SPC and 2 μM of compound **1c** (d) were added.

the aliphatic substituents. Moreover, the activities are increased, in order, among **2** (methyl and propyl), **3a** (ethylcarbamate-spiro), and **1c** (methyl and 4-methoxyphenethyl) in the C-2 substituents in 2*H*-benzopyran. The sulfonyl substituent in the 6-amino position has low activity compared to the acyl substituent (**3a** and **3b**).

The 4-nitrobenzamido-2*H*-benzopyrans (**1c** and **3a**), which have the most potent SPC inhibitory activities (Fig. 3), were subjected to further evaluations including a CYP450 inhibition assay and a hERG binding assay. According to Table 2, 2*H*-benzopyrans **1c** and **3a** showed no hERG binding (IC_{50} > 10 μM) or CYP450 inhibitions (> 10 μM).

Also, we confirmed the anti-SPC effect of 2*H*-benzopyran **1c** on HUVEC tube formation (Fig. 4). After 6 h incubation with or without 10 μM of SPC and 2.5 μM of compound **1c**, 2*H*-benzopyran **1c** inhibited the SPC-induced HUVEC tube formation as shown in Figure 4(d).

Conclusions

In summary, we identified the novel drug-like core 2*H*-benzopyran scaffold as the SPC inhibitory lead compounds, 2,6-difunctionalized 2*H*-benzopyran derivatives from an in-house chemical library screening. The synthetic compounds **1c** and **3a** showed potent SPC inhibitory activities (IC_{50} < 20 nM) and possessed drug-like characteristics in drug-drug interactions and hERG binding assay results. In addition, the anti-SPC effect of 2*H*-benzopyran **1c** was confirmed on HUVEC tube formation assay. Further detailed structure-activity relationship studies are underway, the results of which will be reported in due course.

Experimental Procedure

Chemistry.

General: All chemicals were reagent grade and used as purchased. Reactions were monitored by thin layer chromatography (TLC) analysis using Merck silica gel 60 F-254 thin layer plates. Flash column chromatography was carried out on Merck silica gel 60 (230−400 mesh). The crude products were purified by high throughput chromatography using Isorea One (Biotage). ^{1}H and ^{13}C NMR spectra were recorded in δ units relative to a deuterated solvent as an internal reference using the Bruker 500 MHz NMR instrument. Liquid chromatography/tandem mass spectrometry (LC-MS/MS/Agilent6460 TripleQuad LC/MS) analysis was performed on an electrospray ionization (ESI) mass spectrometer with photodiode-array detector (PDA) detection. The LC-MS/MS area percentage purities of all products were determined by LC peak area analysis (Poroshell 120 EC-C18 column, 4.6 mm × 100 mm, 2.7 Micron; PDA detector at 245 nm; 70% CH_3CN/H_2O).

Representative Procedure for the *N*-(2-Methyl-2*H*-chromen-6-yl)benzamide (1a-1e). 4-Chlorobenzoyl chloride (1.67 g, 9.56 mmol), triethylamine (0.967 g, 9.56 mmol), and DMAP (29.2 mg, 0.239 mmol) were added to a stirred solution of 6-amino-2*H*-benzopyran **5a** (1.41 g, 4,78 mmol) in CH_2Cl_2 (15 mL). The reaction mixture was stirred at room temperature for 5 h. The resulting mixture was extracted with CH_2Cl_2 (3 × 20 mL) and distilled water (20 mL), then the organic layer was dried over $MgSO_4$. After removal of

the solvent *in vacuo*, the residue was purified by a silica gel column (hexane/ethyl acetate, 5:1 v/v) to yield the desired product 4-chloro-*N*-(2-(4-methoxyphenethyl)-2-methyl-2*H*-chromen-6-yl)benzamide (**1a**), which was obtained at 82% (1.70 g, pale oil): ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.80 (s, 1H), 7.72 (dd, *J* = 7.4, 1.8 Hz, 1H), 7.43 (dd, *J* = 6.3, 1.8 Hz, 2H), 7.37 (dtd, *J* = 18.5, 7.3, 1.5 Hz, 2H), 7.21 (dd, *J* = 8.6, 2.6 Hz, 1H), 7.08 (d, *J* = 8.5 Hz, 2H), 6.79 (dd, *J* = 13.9, 8.6 Hz, 3H), 6.39 (d, *J* = 9.9 Hz, 1H), 5.63 (d, *J* = 9.9 Hz, 1H), 3.77 (s, 3H), 2.78–2.60 (m, 2H), 1.96 (dddd, *J* = 49.4, 13.8, 11.8, 5.4 Hz, 2H), 1.43 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 164.26, 157.74, 150.42, 135.27, 134.25, 131.60, 130.61, 130.52, 130.36, 129.23, 127.29, 122.93, 121.41, 121.33, 118.91, 116.36, 113.80, 78.60, 77.28, 77.03, 76.77, 55.28, 43.44, 29.52, 26.57; MS (ESI) *m*/*z* 434.2 ($[M+H]^+$).

2-Fluoro-*N*-(2-(4-methoxyphenethyl)-2-methyl-2*H*-chromen-6-yl)benzamide (1b): Yield, 81% (1.62 g, white solid): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.31 (d, *J* = 15.5 Hz, 1H), 8.17 (t, *J* = 7.9 Hz, 1H), 7.51 (dd, *J* = 13.7, 6.8 Hz, 1H), 7.44 (d, *J* = 2.2 Hz, 1H), 7.31 (t, *J* = 7.6 Hz, 1H), 7.24 (d, *J* = 2.4 Hz, 1H), 7.17 (dd, *J* = 12.3, 8.4 Hz, 1H), 7.09 (d, *J* = 8.4 Hz, 2H), 6.83–6.76 (m, 3H), 6.40 (d, *J* = 9.9 Hz, 1H), 5.64 (d, *J* = 9.9 Hz, 1H), 3.77 (s, 3H), 2.78–2.63 (m, 2H), 1.96 (dtd, *J* = 18.7, 13.8, 5.4 Hz, 2H), 1.44 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 161.24 (d, $^2J_{CF}$ = 34.1 Hz), 160.24, 155.22 (d, $^1J_{CF}$ = 208.6 Hz), 157.74, 150.34, 134.27, 133.57 (d, $^3J_{CF}$ = 9.3 Hz), 132.27, 130.66, 130.30, 129.22, 125.09 (d, $^4J_{CF}$ = 3.1 Hz), 122.97, 121.73, 121.39 (d, $^3J_{CF}$ = 11.1 Hz), 121.36, 119.28, 116.33, 116.09 (d, $^2J_{CF}$ = 25.1 Hz), 113.80, 78.57, 55.28, 43.44, 29.52, 26.56; MS (ESI) *m*/*z* 418.2 ($[M+H]^+$).

***N*-(2-(4-Methoxyphenethyl)-2-methyl-2*H*-chromen-6-yl)-4-nitrobenzamide (1c):** Yield, 78% (1.66 g, yellow solid): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.32 (d, *J* = 8.6 Hz, 1H), 8.01 (d, *J* = 8.6 Hz, 1H), 7.79 (s, 1H), 7.39 (s, 1H), 7.22 (d, *J* = 10.6 Hz, 1H), 7.08 (d, *J* = 8.6 Hz, 1H), 6.80 (t, *J* = 8.4 Hz, 2H), 6.38 (d, *J* = 9.9 Hz, 1H), 5.65 (d, *J* = 9.9 Hz, 1H), 3.78 (s, 3H), 2.79–2.62 (m, 1H), 1.97 (dddd, *J* = 50.3, 13.9, 11.7, 5.4 Hz, 1H), 1.44 (s, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.49, 157.76, 150.77, 149.68, 140.59, 134.16, 130.59, 130.10, 129.21, 128.19, 123.99, 122.74, 121.64, 121.50, 119.19, 116.48, 113.82, 78.75, 77.28, 77.02, 76.77, 55.28, 43.45, 29.50, 26.59; MS (ESI) *m*/*z* 445.2 ($[M+H]^+$).

***N*-(2-(4-Methoxyphenethyl)-2-methyl-2*H*-chromen-6-yl)cyclopropanecarboxamide (1d):** Yield, 70% (1.22 g, white solid): ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.31 (d, *J* = 1.9 Hz, 1H), 7.27 (s, 1H), 7.07 (d, *J* = 8.5 Hz, 3H), 6.80 (d, *J* = 8.6 Hz, 2H), 6.71 (d, *J* = 8.5 Hz, 1H), 6.34 (d, *J* = 9.9 Hz, 1H), 5.60 (d, *J* = 9.9 Hz, 1H), 3.77 (s, 3H), 2.76–2.61 (m, 2H), 1.94 (dtd, *J* = 18.7, 13.8, 5.4 Hz, 2H), 1.45 (ddd, *J* = 12.4, 8.1, 4.6 Hz, 1H), 1.41 (s, 3H), 1.09–1.04 (m, 2H), 0.85–0.79 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.61, 157.72, 149.75, 134.29, 131.18, 130.20, 129.21, 123.03, 121.28, 120.87, 118.66, 116.16, 113.79, 78.42, 77.28, 77.02, 76.77, 55.28, 43.38, 29.49, 26.48, 15.59, 7.75; MS (ESI) *m*/*z* 364.2 ($[M+H]^+$).

***N*-(2-(4-Methoxyphenethyl)-2-methyl-2*H*-chromen-6-yl)pentanamide (1e):** Yield, 68% (1.23 g, white solid): ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.30 (d, *J* = 2.5 Hz, 1H), 7.10–7.04 (m, 4H), 6.80 (d, *J* = 8.6 Hz, 2H), 6.72 (d, *J* = 8.6 Hz, 1H), 6.35 (d, *J* = 9.9 Hz, 1H), 5.60 (d, *J* = 9.9 Hz, 1H), 3.77 (s, 3H), 2.75–2.61 (m, 2H), 2.32 (t, *J* = 7.6 Hz, 2H), 1.94 (dddd, *J* = 18.9, 13.8, 11.9, 5.4 Hz, 3H), 1.70 (dt, *J* = 15.2, 7.6 Hz, 2H), 1.45–1.36 (m, 6H), 0.94 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.21, 157.72, 149.86, 134.29, 130.94, 130.21, 129.21, 123.00, 121.27, 121.03, 118.75, 116.18, 113.79, 78.44, 77.28, 77.03, 76.77, 55.27, 43.39, 37.39, 29.50, 27.80, 26.49, 22.41, 13.84; MS (ESI) *m*/*z* 380.2 ($[M+H]^+$).

Synthesis of the *N*-(2-Methyl-2-propyl-2*H*-chromen-6-yl)-4-nitrobenzamide (2). 4-Nitrobenzoyl chloride (1.58 g, 8.50 mmol), triethylamine (0.860 g, 8.50 mmol), and DMAP (25.9 mg, 0.212 mmol) were added to a stirred solution of 6-amino-2*H*-benzopyran **5b** (1.11 g, 4.25 mmol) in CH_2Cl_2 (15 mL). The reaction mixture was stirred at room temperature for 5 h. The resulting mixture was extracted with CH_2Cl_2 (3 × 20 mL) and distilled water (20 mL), then the organic layer was dried over $MgSO_4$. After removal of solvent *in vacuo*, the residue was purified by a silica gel column (hexane/ethyl acetate, 5:1 v/v) to yield the desired product *N*-(2-methyl-2-propyl-2*H*-chromen-6-yl)-4-nitrobenzamide (**2**), which was obtained at 73% (1.23 g, yellow solid): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.32 (d, *J* = 8.5 Hz, 2H), 8.01 (d, *J* = 8.5 Hz, 2H), 7.78 (s, 1H), 7.36 (d, *J* = 1.4 Hz, 1H), 7.20 (dd, *J* = 8.4, 1.7 Hz, 1H), 6.75 (d, *J* = 8.5 Hz, 1H), 6.32 (d, *J* = 9.9 Hz, 1H), 5.61 (d, *J* = 9.9 Hz, 1H), 1.75–1.61 (m, 2H), 1.51–1.41 (m, 2H), 1.39 (s, 3H), 0.92 (t, *J* = 7.3 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.49, 150.89, 149.66, 140.61, 130.98, 129.93, 128.18, 123.98, 122.30, 121.60, 121.57, 119.14, 116.41, 79.05, 77.27, 77.02, 76.77, 43.62, 26.51, 17.25, 14.41; MS (ESI) *m*/*z* 353.2 ($[M+H]^+$).

Representative Procedure for the Spiro-benzopyran (3a-3b): 4-Nitrobenzoyl chloride (0.778 g, 4.19 mmol), triethylamine (0.579 g, 5.72 mmol), and DMAP (46.5 mg, 0.381 mmol) were added to a stirred solution of *N*-[ethyl-carbamate-spiro(2*H*-1-benzopyran-2,4-piperidine)-6-yl]amine (**6**) (1.10 g, 3.81 mmol) in CH_2Cl_2 (10 mL) and stirred at room temperature conditions for 7 h. The resulting mixture was extracted with CH_2Cl_2 (3 × 20 mL) and distilled water (20 mL), then the organic layer was dried over $MgSO_4$. After removal of the solvent *in vacuo*, the residue was purified by a silica gel column (methanol/dichloromethane, 1:10 v/v) to yield the desired product **3a**, which was obtained at 60% (1.00 g, light yellow solid): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.32 (d, *J* = 8.7 Hz, 2H), 8.03 (d, *J* = 8.6 Hz, 2H), 7.99 (s, 1H), 7.41 (d, *J* = 1.7 Hz, 1H), 7.28–7.23 (m, 1H), 6.84 (d, *J* = 8.6 Hz, 1H), 6.39 (d, *J* = 9.8 Hz, 1H), 5.59 (d, *J* = 9.8 Hz, 1H), 4.15 (q, *J* = 7.1 Hz, 2H), 4.01–3.82 (m, 2H), 3.32 (d, *J* = 0.6 Hz, 2H), 1.99 (d, *J* = 12.6 Hz, 2H), 1.66–1.53 (m, 2H), 1.27 (t, *J* = 5 Hz, 3H); ^{13}C NMR (125 MH, $CDCl_3$) δ 163.60, 155.63, 149.69, 149.68, 140.49, 130.78, 130.02, 128.27, 123.95, 123.46, 122.18, 121.89, 119.45, 116.81, 74.94, 61.41, 39.22, 34.78, 14.72; MS (ESI) *m*/*z* 438.2 ($[M+H]^+$).

Ethyl Piperidine-1'-carboxylate-4'-spiro-2-6-(naphthal-

ene-1-sulfonamido)-2*H*-benzopyran (3b). Yield, 65% (1.19 g, beige solid): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.29 (d, *J* = 1.5 Hz, 1H), 7.88 (t, *J* = 8.2 Hz, 2H), 7.71 (dd, *J* = 8.7, 1.8 Hz, 1H), 7.60 (dtd, *J* = 16.1, 7.0, 1.0 Hz, 2H), 6.81 (d, *J* = 2.5 Hz, 1H), 6.77–6.70 (m, 2H), 6.62 (d, *J* = 8.5 Hz, 1H), 6.24 (d, *J* = 9.8 Hz, 1H), 5.50 (d, *J* = 9.8 Hz, 1H), 4.13 (dt, *J* = 14.2, 5.5 Hz, 2H), 3.86 (d, *J* = 2.1 Hz, 2H), 3.25 (s, 2H), 1.91 (d, *J* = 12.9 Hz, 2H), 1.64 (s, 1H), 1.55 (td, *J* = 13.6, 4.8 Hz, 2H), 1.27–1.22 (m, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 155.60, 150.50, 136.00, 134.86, 132.03, 129.79, 129.37, 129.28, 129.27, 128.87, 128.82, 127.89, 127.50, 124.75, 123.20, 122.40, 122.35, 122.28, 116.93, 74.94, 61.40, 39.15, 34.76, 14.69; MS (ESI) *m*/*z* 477.1 ([M-H]$^-$).

Biology.

Reagents: SPC was purchased from Matreya Inc. (Japan). [^{3}H]-thymidine was from Amershan and unspecified reagents were from Sigma.

Cell Culture. NIH3T3 (murine fibroblast) cells were obtained from American Type Culture Collection (Manassas, VA) and were maintained in DMEM (Gibco) with 10% fetal bovine serum, 100 U/mL penicillin, and 1% streptomycin. The cells were cultured in a 5% CO_2 incubator with 95% humidity at 37 ºC.

SPC-induced Proliferation Assay. Cells were seeded in 96-well plates at 5×10^3 cells per well and stabilized overnight. The cells were treated with test compounds at indicated concentrations for 30 min and supplemented with SPC. The cells were incubated for 24 h and a 1/10 volume of [^{3}H]-thymidine (final 0.5 μCi/well) was added and further incubated for 24 h. The medium was aspirated and the cells were thoroughly washed three times with PBS. Subsequently, a scintillation cocktail was added and a radioisotope incorporated into the cells was measured in Trilux (PerkinElmer).

MTT Assay. Cytotoxic effect was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) viability assay in NIH3T3. The MTT assay is a standard colorimetic assay measuring the activity of enzymes that reduce MTT to insoluble purple formazan crystals. The cells were grown in each media (NIH 3T3, DMEM with 10% FBS; HUVEC, M199 with 20% FBS, 100 ng/mL of heparin, 5 ng/mL of bFGF) and seeded into a 96-well plate at a density of 5×10^3 cells/well and treated with 50, 10, 2, 0.4, 0.08, 0.016 μM of each compound for 48 h. The 1/10 volume of MTT stock (5 mg/mL in PBS) was added and further incubated for 1 h. The media were aspirated and 100 μL of DMSO were added to each well. After 10 min incubation, the absorbance was measured at 570 nm.

Tube Formation Assay. Matrigel (10 mg protein/mL, Clontech, MA, USA) 40 μL was pipetted into a 96-well culture plate and polymerized for 1 h at 37 °C. HUVECs were harvested after trypsin-EDTA treatment, re-suspended in M199 and then plated onto a layer of Matrigel at a density of 2×10^4 cells/well, followed by the addition of compounds (10, 2 and 0.4 μM). After the Matrigel cultures were incubated at 37 °C for 18-24 h, the cultures were photographed (40x). Each dose of control or test compound was assayed in duplicate and the assays were repeated three times.

CYP450 Inhibition Assay. Cytochrome P-450 enzyme inhibition was measured using the P450-glo™ assay kit (V9770, 9790, 9880, 9890, and V9800 of Promega, WI) according to the protocols provided by the manufacturer. First, the compounds were diluted into 4x the final test concentrations (4x sample). Each CYP membrane and the corresponding luciferin-tagged substrate were diluted with water (4x CYP). Equal volume (6.25 μL) of the 4x sample and 4x CYP were mixed in a 384-well white plate and preincubated at room temperature for 10 min. Then, the plate was stored for 20-30 min after adding 12.5 μL of a 2x NADPH generation system. Luminescence was detected in Fusion-alpha® (PerkinElmer, MA) after 20 min stabilization with a luciferin detection reagent.

hERG Binding Assay. [^{3}H]Astemizole and human ERG K^+ channel expressed in HEK-293 cells were purchased from PerkinElmer. Assays were performed in 200 μL of 50 mM Hepes (pH 7.4), 60 mM KCl, 0.1% BSA, 4 nM [^{3}H]-astemizole with 2.5 μg of membranes. Assay mixtures were incubated for 1 h at room temperature and filtered through a Filtermat-A pre-soaked in 0.3% PEI. The signal was detected with a MicroBeta® (PerkinElmer). Nonspecific binding was determined in the presence of 0.1 μM astemizole. Competition binding studies were carried out with 5-6 varied concentrations of the test compounds run in duplicate tubes, and isotherms from two assays were calculated.

Data Analysis. Experimental data from dose-response curves were analyzed by a nonlinear regression analysis with GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA) and the IC_{50} values were obtained. Statistical analyses for comparisons of the differences in means of the two groups were carried out using a student's t-test.

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