Discovery of Novel Mesangial Cell Proliferation Inhibitors Using a Three-Dimensional Database Searching Method

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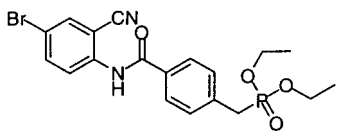
A three-dimensional pharmacophore model of mesangial cell (MC) proliferation inhibitors was generated from a training set of 4-(diethoxyphosphoryl)methyl-*N*-(3-phenyl-[1,2,4]thiadiazol-5-yl)benzamide, **2**, and its derivatives using the Catalyst/HIPHOP software program. On the basis of the in vitro MC proliferation inhibitory activity, a pharmacophore model was generated as seven features consisting of two hydrophobic regions, two hydrophobic aromatic regions, and three hydrogen bond acceptors. Using this model as a three-dimensional query to search the Maybridge database, structurally novel 41 compounds were identified. The evaluation of MC proliferation inhibitory activity using available samples from the 41 identified compounds exhibited over 50% inhibitory activity at the 100 nM range. Interestingly, the newly identified compounds by the 3D database searching method exhibited the reduced inhibition of normal proximal tubular epithelial cell proliferation compared to a training set of compounds.

Introduction

Mesangial cells (MC) serve a number of functions in the renal glomerular capillary including structural support of the capillary tuft, modulation of glomerular hemodynamics, and a phagocytic function allowing removal of macromolecules and immune complexes. The proliferation of mesangial cells (MC) is a prominent feature of glomerular disease including IgA nephropathy, membranoproliferative glomerulonephritis, lupus nephritis, and diabetic nephropathy.[1] In experimental animal models of nephritis, MC proliferation frequently precedes and is linked to the increase of extracellular matrix in the mesangium and glomerulosclerosis.[2,3] Reduction of MC proliferation in glomerular disease models by the treatment with heparin,[4] low-protein diet,[5] or antibodies to platelet-derived growth factor (PDGF)[6] has been shown to reduce extracellular matrix expansion and glomerulosclerotic changes. Therefore, MC proliferation inhibitors may offer therapeutic opportunities for the treatment of proliferative glomerular disease. It is also known that the MC proliferation is inhibited by many kinds of pharmacological drugs, e.g., ACE inhibitors,[7] leukotrien D4 (LTD_4) antagonists,[8] PDGF inhibitors,[9] matrix metalloproteinase (MMP) inhibitors,[10] HMG-CoA inhibitors,[11] cyclooxygenase inhibitors,[12] cyclin-dependent kinase antagonists,[13] and so on. However, selective MC proliferation inhibitors against normal cells at the pharmacological range have not been reported.

The antilipidemic agent, diethyl 4-(diethoxyphosphoryl)methyl-*N*-(4-bromo-2-cyanophenyl)benzamide, NO-1886, **1**, has a unique profile that increases HDL-C by activation of lipoprotein lipase (LPL).[14−16] In the pharmacological profiling study of **1**, the significant inhibitory activity of in vitro MC proliferation was found at 10 μM concentration. The biological screening of our in-house benzylphosphonate library similar to **1** yielded four potent MC proliferation inhibitors, 4-(diethoxyphosphoryl)methyl-*N*-(3-phenyl-[1,2,4]thiadiazol-5-yl)benzamide, **2**, and its derivatives, **3**, **4**, and **5** (Figure 1), which indicated significant MC proliferation inhibitory activity at 100 nM concentration.



NO-1886, **1**

To design novel MC proliferation inhibitors, a three-dimensional (3D) pharmacophore model using the Catalyst software program would be useful to identify the structural requirements for the inhibitory activity in this series.[17] The obtained pharmacophore model may also be useful for 3D queries to search databases of proprietary and/or commercially available compounds.[18] In this paper, the generation of a pharmacophore model for MC proliferation inhibitors from a training set of **2**−**5** using Catalyst/HIPHOP, the database search using an obtained pharmacophore model and the pharmacological results of the identified compounds, is discussed.

Results and Discussion

Four MC proliferation inhibitors, 4-(diethoxyphosphoryl)methyl-*N*-(3-phenyl-[1,2,4]thiadiazol-5-yl)benzamide, **2**, and its derivatives, **3**, **4**, and **5** (Figure 1), which indicated significant MC proliferation inhibitory activity at 100 nM concentration (Table 1) were selected as a training set of compounds for the pharmacophore

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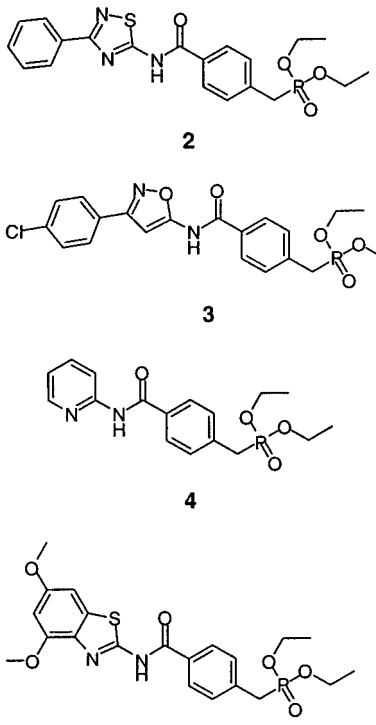


Figure 1. Training set of four MCP inhibitors used to generate the pharmacophore model.

Table 1. Dose-Dependent MC Proliferation Inhibitory Activities and Fit Values

	MC proliferation inhibition (%)[a]			
compd	10 μM	1 μM	0.1 μM	fit
1	56	ND[b]	ND[b]	ND[b]
2	108	85	62	6.81
3	94	91	69	7.00
4	68	65	62	4.97
5	108	59	59	5.28
6	112	105	90	5.72
7	82	73	68	4.67
8	80	74	72	5.15
9	66	60	49	5.00

[a] See Experimental Section. [b] ND = not determined.

analysis. The compounds in the training set possessed diverse heterocycles to give structural requirements for the pharmacophore model generation. Conformation analysis of the training sets was identified up to 250 conformers for each molecule within 20 kcal/mol conformational energy above the lowest-energy conformation. Using the training sets with the multiconformation, the pharmacophore model of MC proliferation inhibitors in this series was generated as a seven-feature model containing two hydrophobic regions, two hydrophobic aromatic regions, and three hydrogen bond acceptors. Compound **3** indicated the highest fit with the Catalyst-generated pharmacophore model (fit = 7.00) as described in Figure 2. The other sets, **2**, **4**, and **5**, also showed the excellent fit values (4.97−6.81) with the pharmacophore model as listed in Table 1.

To identify structurally novel MC proliferation inhibitors, the Catalyst-generated pharmacophore model of MC proliferation inhibitors was used as a 3D query to search the Maybridge 3D database and results in the identification of a total of 41 structurally novel compounds (hits). Four compounds, **6** (BTB07897), **7** (BTB09702), **8** (RJF01928), and **9** (RJF01934), were selected from the identified 41 compounds because of availability for the MC proliferation assay and for possessing >4.50 of fit values. The structures of four compounds, **6**−**9**, were different from that of a training set and other known MC proliferation inhibitors (Figure 3). Compound **6** indicated the highest fit with the Catalyst-generated pharmacophore model (fit = 5.72) as described in Figure 4. The other compounds, **7**−**9**, also showed excellent fit values (4.67−5.15) with the pharmacophore model.

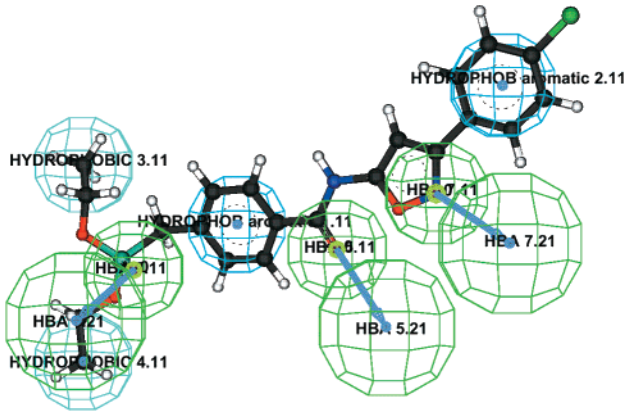


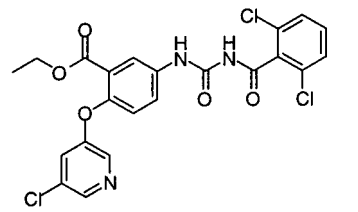
Figure 2. Best conformation of **3** flexibility fit to the Catalyst-generated pharmacophore model of MC proliferation inhibitors.

After the in vitro evaluation, all four compounds exhibited potent MC proliferation inhibitory activities at 100 nM concentration as listed in Table 1. The 100% hit ratio obtained from the focused compounds of the Maybridge database is significant improvement compared to the random screening of the structurally similar compounds. The random screening of our in-house benzylphosphonate library similar to **1** identified four compounds, which exhibited significant MC proliferation inhibitory activities at 100 nM concentration.

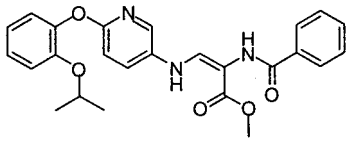
The compounds, which exhibited MC proliferation inhibitory activities, were done via the RPTEC proliferation assay in order to evaluate the cell toxicity against normal cells. The RPTEC proliferation inhibitory activities (%) at the dose−response concentration are listed in Table 2. Interestingly, the compounds newly identified by the 3D database searching method exhibited the weak inhibition of RPTEC proliferation compared to a training set of compounds. In particular, compound **7**, which showed no RPTEC proliferation inhibitory activity at 10 μM concentration, is the most favorable compound as a selective MC proliferation inhibitor.

Conclusion

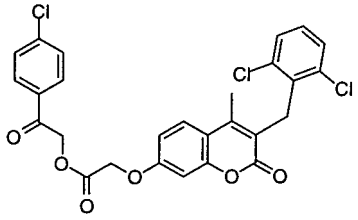
To identify novel MC proliferation inhibitors, a three-dimensional pharmacophore model was generated using a training set of novel MC proliferation inhibitors, 4-(diethoxyphosphoryl)methyl-*N*-(3-phenyl-[1,2,4]thiadiazol-5-yl)benzamide, **2**, and its derivatives, **3**−**5**. The generated pharmacophore model containing two hydrophobic regions, two hydrophobic aromatic regions, and three hydrogen bond acceptors identified 41 compounds from the Maybridge database. Four available com-



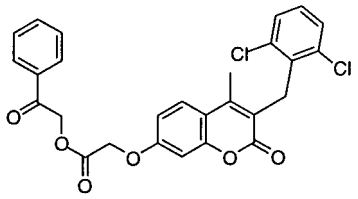
BTB07897, **6**



BTB09702, **7**



RJF01928, **8**



RJF01934, **9**

Figure 3. Four compounds identified by searching the Maybridge database using the Catalyst-generated pharmacophore model of MC proliferation inhibitors.

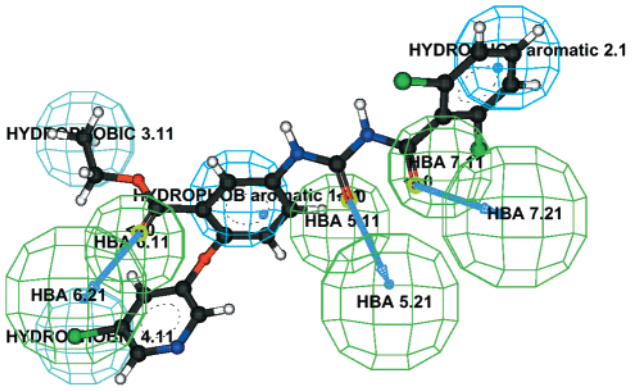


Figure 4. Best conformation of **6** flexibility fit to the catalyst-generated pharmacophore model of MC proliferation inhibitors.

pounds with fit values of >4.5 exhibited significant MC proliferation inhibitory activities at 100 nM concentration. Interestingly, the newly identified compounds showed weaker inhibition of RPTEC proliferation compared to a training set of compounds. Finally, the Catalyst-generated pharmacophore model of MC proliferation inhibitors in this series was useful in the rational design and identification of other novel MC proliferation inhibitors without cell toxicity.

Table 2. Dose-Dependent Renal Proximal Tubular Epithelial Cell (RPTEC) Proliferation Inhibitory Activities

	RPTEC proliferation inhibition (%)[a]		
compd	10 μM	1 μM	0.1 μM
1	77	ND[b]	ND[b]
2	96	61	33
3	99	88	30
4	60	32	23
5	103	39	33
6	96	32	−1
7	−8	−12	2
8	21	6	16
9	31	24	35

[a] See Experimental Section. [b] ND = not determined.

Experimental Section

Synthesis. 4-(Diethoxyphosphoryl)methyl-*N*-(3-phenyl-[1,2,4]-thiadiazol-5-yl)benzamide and its derivatives, **2**−**5**, which were selected for a training set of Catalyst analysis, were prepared using methodology that has been published previously.[19] ^{1}H NMR spectra were recorded on a JEOL JNM-AL400 FT NMR (400 MHz) spectrometer with tetramethylsilane as an internal standard. Mass spectra were measured on a JEOL JMS-700 mass spectrometer, equipped with an electrospray interface (EI). Experiments were done in positive ionization mode. Melting points were determined with a YAMATO MP-21 capillary melting point apparatus. Elemental analyses were preformed by the analytical department of Wako Pure Chemical Industries, Ltd. (Japan), and are within ±0.4% of theoretical values.

4-(Diethoxyphosphoryl)methyl-*N*-(3-phenyl-[1,2,4]thiadiazol-5-yl)benzamide (2): mp 209−211 °C; ^{1}H NMR (DMSO-d_6) δ 1.17 (t, J = 5.2 Hz, 6H), 3.39 (d, J = 22.4 Hz, 4H), 3.98 (q, J = 7.2 Hz, 4H), 7.49−7.57 (m, 5H), 8.14 (d, J = 8.0 Hz, 2H), 8.21 (d, J = 8.0 Hz, 2H), 13.6 (br, 1H); MS (EI) m/z 431. Anal. ($C_{20}H_{22}N_3O_4PS$) C, H, N.

4-(Diethoxyphosphoryl)methyl-*N*-[3-(4-chlorophenyl)-isoxazol-5-yl]benzamide (3): mp 225−226 °C; ^{1}H NMR (DMSO-d_6) δ 1.17 (t, J = 5.2 Hz, 6H), 3.37 (d, J = 22.0 Hz, 4H), 4.00 (q, J = 6.8 Hz, 4H), 6.97 (s, 1H), 7.46 (d, J = 6.0 Hz, 2H), 7.59 (d, J = 6.8 Hz, 2H), 7.92 (d, J = 8.8 Hz, 2H), 8.01 (d, J = 7.6 Hz, 2H), 12.1 (br, 1H); MS (EI) m/z 448. Anal. ($C_{21}H_{22}ClN_2O_5P$) C, H, N.

4-(Diethoxyphosphoryl)methyl-*N*-[pyridin-2-yl]benzamide (4): mp 94−96 °C; ^{1}H NMR (DMSO-d_6) δ 1.19 (t, J = 7.2 Hz, 6H), 3.34 (d, J = 22.0 Hz, 4H), 3.97 (q, J = 6.0 Hz, 4H), 7.16 (t, J = 4.8 Hz, 1H), 7.41 (d, J = 8.4 Hz, 2H), 7.84 (t, J = 6.8 Hz, 1H), 7.98 (d, J = 8.4 Hz, 2H), 8.18 (d, J = 8.4 Hz, 1H), 8.39 (d, J = 4.0 Hz, 1H), 10.7 (br, 1H); MS (EI) m/z 348. Anal. ($C_{17}H_{21}N_2O_4P$) C, H, N.

4-(Diethoxyphosphoryl)methyl-*N*-[4,6-dimethoxybenzothiazol-2-yl]benzamide (5): mp 210−211 °C; ^{1}H NMR (DMSO-d_6) δ 1.19 (t, J = 7.2 Hz, 6H), 3.37 (d, J = 22.0 Hz, 4H), 3.82 (s, 3H), 3.91 (s, 3H), 3.96 (q, J = 6.0 Hz, 4H), 6.62 (s, 1H), 7.16 (s, 1H), 7.45 (d, J = 8.0 Hz, 2H), 8.09 (d, J = 8.4 Hz, 2H), 12.8 (br, 1H); MS (EI) m/z 464. Anal. ($C_{21}H_{25}N_2O_6PS\cdot H_2O$) C, H, N.

Catalyst Pharmacophore Construction and Database Searching. Calculations were performed using a Silicon Graphics Indigo 2 under IRIX 6.3. The generation of the pharmacophore model for MC proliferation inhibitors and a 3D query of Maybridge database using the generated pharmacophore model were accomplished using Catalyst, version 3.1.[20] The pharmacophore model (in Catalyst/HIPHOP called a hypothesis) consists of a set of chemical features necessary for the biological activity of the compounds arranged in a three-dimensional space.[21] Hydrophobic regions, hydrophobic aromatic regions, and hydrogen bond acceptors were used for the chemical features in this calculation. Hydrophobic regions are located at centroids of the hydrophobic ligand atom. Hydrophobic aromatic regions are placed at centroids of the aromatic ring. Hydrogen bond acceptors are characterized by electronic lone pairs of nitrogen, oxygen, or sulfur atoms. The Maybridge 3D-coordinate database (47 045 compounds) was supplied from Molecular Simulations, Inc. (MSI) with Catalyst software. The

compounds identified by searching the Maybridge database were obtained from Maybridge Co. Ltd., Trevillett, Tintagel, Cornwall PL3 OHW, U.K.

Ethyl 2-(5-chloropyridin-3-yloxy)-5-[3-(2,6-dichlorobenzoyl)ureido]benzoate (6): mp 196−198 °C; ^{1}H NMR (DMSO-d_6) δ 1.08 (t, J = 6.8 Hz, 3H), 4.16 (q, J = 6.8 Hz, 2H), 7.32 (d, J = 8.4 Hz, 2H), 7.40 (s, 1H), 7.56−7.59 (m, 3H), 7.9 (br, 1H), 8.24 (br, 2H), 8.35 (s, 1H), 10.5 (br, 1H), 11.6 (br, 1H); MS (EI) m/z 507. Anal. ($C_{22}H_{16}Cl_3N_3O_5 \cdot 0.5H_2O$) C, H, N.

Methyl 2-benzoylamino-3-[6-(2-isopropoxyphenoxy)-pyridin-3-ylamino]acrylate (7): recrystallization from CH_2Cl_2/n-hexane gave colorless needles, mp 122−124 °C; ^{1}H NMR (DMSO-d_6) δ 1.04 (d, J = 6.4 Hz, 6H), 3.62 (s, 3H), 4.48 (q, J = 6.4 Hz, 2H), 6.89 (d, J = 8.8 Hz, 1H), 6.94 (dt, J = 1.6 and 7.6 Hz, 1H), 7.08−7.16 (m, 3H), 7.48 (t, J = 14.0 Hz, 2H), 7.56 (t, J = 6.8 Hz, 1H), 7.69 (dd, J = 2.8 and 6.0 Hz, 1H), 7.83 (d, J = 12.8 Hz, 1H), 7.95 (d, J = 3.2 Hz, 1H), 8.01 (d, J = 7.6 Hz, 2H), 8.84 and 8.88 (2 singlets, 1H), 9.14 (br, 1H); MS (EI) m/z 447. Anal. ($C_{25}H_{25}N_3O_5$) C, H, N.

2-(4-Chlorophenyl)-2-oxoethyl 3-(2,6-dichlorobenzyl)-4-methyl-2-oxo-2*H*-chromen −7-yloxyacetate (8): mp 169−170 °C; ^{1}H NMR (DMSO-d_6) δ 2.36 (s, 3H), 4.23 (s, 2H), 5.12 (s, 2H), 5.64 (s, 2H), 7.01 (d, J = 2.4 Hz, 1H), 7.03 (s, 1H), 7.27 (t, J = 8.0 Hz, 1H), 7.42 (d, J = 8.0 Hz, 2H), 7.63 (d, J = 8.4 Hz, 2H), 7.74 (d, J = 9.6 Hz, 1H), 7.98 (d, J = 8.4 Hz, 2H); MS (EI) m/z 544. Anal. ($C_{27}H_{19}Cl_3O_6$) C, H, N.

2-Oxo-2-phenylethyl 3-(2,6-dichlorobenzyl)-4-methyl-2-oxo-2*H*-chromen-7-yloxy-acetate (9): mp 157−158 °C; ^{1}H NMR (DMSO-d_6) δ 2.36 (s, 3H), 4.24 (s, 2H), 5.13 (s, 2H), 5.65 (s, 2H), 7.01−7.04 (m, 2H), 7.27 (t, J = 8.8 Hz, 1H), 7.43 (d, J = 8.4 Hz, 2H), 7.56 (t, J = 7.6 Hz, 2H), 7.72 (t, J = 7.2 Hz, 1H), 7.74 (d, J = 9.6 Hz, 1H), 7.97 (d, J = 7.2 Hz, 2H); MS (EI) m/z 510. Anal. ($C_{27}H_{20}Cl_2O_6$) C, H, N.

In Vitro Inhibitory Assays of MC and RPTEC Proliferation. The in vitro MC proliferation inhibitory activity of the compounds was determined by measuring the incorporation of [^{3}H]-thymidine into the acid-insoluble fraction of normal human mesangial cells.[22,23] The human glomerular mesangial cells (lot no. 7F0255, Clonetics) were plated on a 75 cm^2 flask (Greiner) and maintained in MCDB131 medium supplemented with amphotericin B at 50 ng/mL, gentamycin at 50 μg/mL, and 5% fetal bovine serum (FBS, BioWhittaker). The MCs were harvested with trypsin-EDTA solution and passaged weekly. The MCs were plated at 2×10^4 cells/well in 24 well plates and incubated for 3 days in the medium described above. The medium was replaced with serum-free MCDB131 medium containing the antibiotics, and the incubation continued for another 4 days. Media was then removed, MCs were incubated with Dulbecco's modified Eagle medium (DMEM) as blank, DMEM containing 2% FBS alone as control, or DMEM containing 2% FBS plus various concentrations of compounds for 18 h in the presence of 1 μCi/mL of [^{3}H]thymidine (5 Ci/mL, Amersham Pharmacia Biotech). The cells were washed and then treated with ice-cold 10% trichloroacetic acid. Counts remaining in each well representing [^{3}H]thymidine incorporation as measure of DNA synthesis were measured in a liquid scintillation counter (Tri-Carb 2500TR, Pachard Instruments). Four wells were served for each concentration of a compound. Proliferation inhibition was expressed as [mean counts in control − mean counts in testing sample]/[mean counts in control − mean counts in blank].

The renal proximal tubular epithelial cell (RPTEC) proliferation assay was essentially identical with the MC proliferation assay using RPTECs (lot no. 6F0534, Clonetics Cell System). Selectivity of proliferation inhibition to each cells was expressed as the inhibitory activities (%) at the same concentration of compound.

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