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Discovery of novel 2-hydroxydiarylamide derivatives as TMPRSS4 inhibitors

Sunghyun Kang [a,†], Hye-Jin Min [b,†], Min-Seo Kang [c], Myung-Geun Jung [c], Semi Kim [b,*]

[a] Biomedical Proteomics Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejon 305-806, Republic of Korea
[b] Immunotherapy Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejon 305-806, Republic of Korea
[c] Division of Drug Discovery Research, Korea Research Institute of Chemical Technology, Daejon 305-600, Republic of Korea

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ABSTRACT

TMPRSS4 is a novel type II transmembrane serine protease that has been implicated in the invasion and metastasis of colon cancer cells. In this study, a novel series of 2-hydroxydiarylamide derivatives were synthesized and evaluated for inhibiting TMPRSS4 serine protease activity and suppressing cancer cell invasion. These derivatives demonstrated good inhibitory activity against TMPRSS4 serine protease, which correlated with the promising anti-invasive activity of colon cancer cells overexpressing TMPRSS4.

The invasive nature of tumor cells plays an important role in cancer metastasis. Cancer cell invasion is a complex process that occurs in the following three steps: altered cell adhesion to the extracellular matrix; degradation of matrix proteins, releasing cells from the primary tumor mass; and cell migration through the newly created matrix defect.[1] The dysregulation of proteases is a hallmark of cancer. Extracellular proteolytic enzymes, including matrix metalloproteinases (MMPs) and serine proteases, contribute to tumor cell invasion and metastasis through both direct proteolytic activity and the regulation of cellular signaling and functions.[1–3]

TMPRSS4, initially referred to as TMPRSS3, is a novel type II transmembrane serine protease (TTSP) that is highly expressed on the cell surface of pancreatic, thyroid, lung, colon, and other cancer tissues.[4–6] Most TTSPs have been implicated in tumor development and progression, mainly based on their dysregulated expression.[7,8] Previously, we demonstrated that TMPRSS4 acts as an important mediator of human tumor cell invasion, migration, and metastasis by facilitating the epithelial-mesenchymal transition, including the downregulation of E-cadherin, a cell–cell adhesion molecule.[9] We also showed that the overexpression of TMPRSS4 potentially activates the FAK/MAPK pathway. TMPRSS4 has also been shown to play a role in the activation of the PI3K/Akt signaling pathway.[6] Furthermore, TMPRSS4 expression was significantly higher in human colorectal cancer tissues from metastatic advanced stages than early stages.[6] These findings suggest that TMPRSS4 is directly involved in the processes of invasion and migration that are characteristic of metastatic malignancy whereas TMPRSS4 does not confer a growth advantage to colon cancer cells. Notably, we observed that TMPRSS4 induces cancer cell invasion in a manner that is dependent on serine proteolytic activity.[9] Recently, TMPRSS4 serine proteolytic activity was determined to be involved in the processing of pro-urokinase-type plasminogen activator into its active form to promote cancer cell invasion (S.K., unpublished results).

TMPRSS4 may be a potential therapeutic target for cancer treatment, particularly for reducing cancer invasion and metastasis. To date, no specific TMPRSS4 inhibitors have been reported. Given the role of TMPRSS4 serine proteolytic activity in tumor cell invasion, the inhibition of this activity could be a potential new approach to developing cancer therapeutics.

Screening of our internal compound library against TMPRSS4 serine protease activity[10] yielded several classes of compounds, including 2-hydroxydiarylamide. *N*-(3,5-bis(trifluoromethyl)-phenyl)-5-chloro-2-hydroxybenzamide (**2a**) exhibited relatively potent inhibitory activity (IC_{50} = 11 μM) against TMPRSS4.

The 2-hydroxydiarylamide series has been reported to have a wide variety of interesting biological properties, including fungicidal, viral and bactericidal activities.[11–16] In particular, compound **2a** (IMD-0354) is a selective IκB kinase (IKK) β inhibitor that has been reported to be effective in acute and subacute inflammatory diseases. In addition, this compound is safe in vitro and in vivo[17] and is currently used in clinical trials to treat patients with atopic

* Corresponding author. Tel.: +82 42 860 4228; fax: +82 42 860 4149.
E-mail address: semikim@kribb.re.kr (S. Kim).
† These authors contributed equally to this work.

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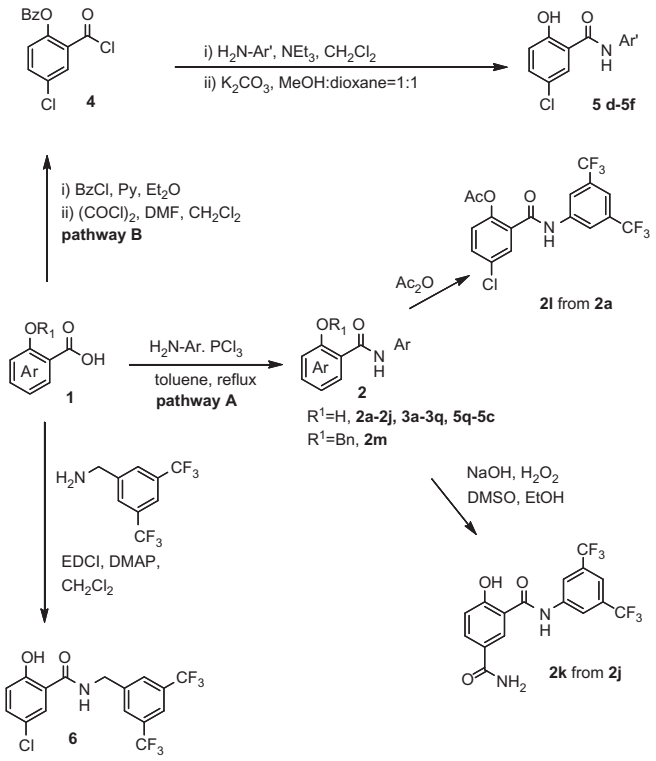
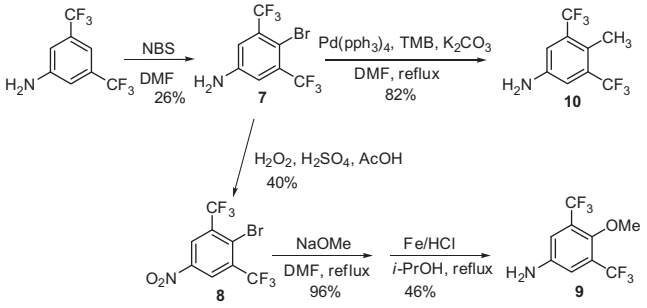


Scheme 1. Synthesis of 2-hydroxydiarylamides.



Scheme 2. Synthesis of anilines.

dermatitis. However, the inhibitory activity of the 2-hydroxydiarylamide series against any serine proteases involved in cancer has not been reported.

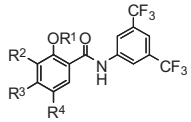
As an initial study, structure–activity relationships (SAR) of derivatives of compound **2a** against TMPRSS4 serine protease activity were determined by synthesizing 2-hydroxydiarylamides with chemical modifications of each aryl group.[16]

The general synthetic pathways of 2-hydroxydiarylamide derivatives of compound **2a** are outlined in Scheme 1. Direct amide coupling reaction of commercially available 2-hydroxyaryl acid **1** and arylamine in the presence of PCl_3 in toluene afforded the corresponding 2-hydroxydiarylamides in moderate to good yields (pathway A). For the synthesis of compounds **3n–p**, bromination of 3,5-bis(trifluoromethyl)benzenamine with NBS in DMF gave 4-bromo-1,3-bis(trifluoromethyl)benzenamine (**7**) in 26% yields, along with 2-bromo-3,5-bis(trifluoromethyl)-benzenamine (47%) as a regioisomer of compound **7** (Scheme 2).[18,19] The amino group of compound **7** was oxidized to a nitro group with H_2O_2 to yield the nitro derivative **8**. After converting bromine in compound **8** to a methoxy group, the reduction of the nitro group was performed in the presence of iron powder under acidic conditions to get the unknown aniline **9**. Compound **10** was synthesized using palladium-catalyzed Suzuki–Miyaura coupling of compound **7** with trimethylboroxine (TMB).[20,21] The acetylation of compound **2a** with acetic anhydride provided of compound **2l** in 46% yield. In addition, the aryl nitrile **2k** was easily hydrolyzed into compound **2j**. On the other hand, when we tried to synthesize *N*-heteoarylamide **5** using synthetic pathway A, the overall yield of the desired products were relatively lower than that of pathway B. According to reaction pathway B, the benzoylation of compound **1** and sequential treatment with oxalyl chloride afforded 4-chloro-2-(chlorocarbonyl)phenyl benzoate (**4**) as a key intermediate. Compound **4** was then coupled with heteroaryl amine under basic conditions followed by hydrolysis to yield compounds **5d–f**. Salicylic acid **1** activated with EDCI coupled with 3,5-bis(trifluoromethyl)benzamine provided compound **6**. The synthetic procedures and spectral analysis data of arylamides are summarized in the Supplementary data. These new derivatives were evaluated fluorometrically for the inhibition of TMPRSS4 serine protease activity. The results are shown in Tables 1–4. AEBSF, a general serine protease inhibitor, exhibited an IC_{50} value of 39 μM, which is greater than compound **2a**.

First, we studied the effect of modifying substituents on the 2-hydroxyphenylamido group of compound **2a** as summarized in Table 1. In compound **2b**, containing a hydrogen in place of chloride at R^4 position, we observed a significant reduction of inhibitory activity against TMPRSS4 serine protease. Moreover, halogenated isomeric 2-hydroxyarylamides (**2c–f**) of compound **2a** did not improve inhibition activity against TMPRSS4 serine protease. In the electron-donating group, substituting to Me and OMe group from chloride at R^4 (**2g** and **h**) reduced inhibitory activities. In the introduction of polar group at R^4 position (see *CLogP* values in Table S1), NO_2 derivative **2i** showed similarly inhibition to **2a**. The activities of **2l** and **m** indicated that the hydroxyl group of 2-hydroxyarylamide is important for inhibition.

After understanding the importance of the 4-chloro-2-hydroxyphenyl amido group for potent inhibition, we attempted to functionalize the phenyl moiety of compound **2a** (Table 2). The unsubstituted phenyl derivative **3a** (R'^1–R'^4 = H) exhibited weak inhibitory activity against the TMPRSS4 serine protease. Compared with substitution with Cl, Br, Me or OMe at R'^1- and R'^3-positions instead of bis-CF_3 of compound **2a**, the order of inhibitory activity was as follows: CF_3-Br-Ph (**3e**) > CF_3-OMe-Ph (**3d**) > di-Cl-Ph (**3c**) > di-Me-Ph (**3b**). In CF_3-containing derivatives, the substitution of the

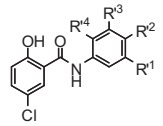
Table 1
Inhibition of TMPRSS4 serine protease activity by 2-hydroxydiarylamides **2a–m**



Compound	R^1	R^2	R^3	R^4	IC_{50} (μM)
2a	H	H	H	Cl	11
2b	H	H	H	H	>100
2c	H	H	H	Br	13
2d	H	H	H	F	29
2e	H	Cl	H	H	12
2f	H	H	Cl	H	12
2g	H	H	H	Me	28
2h	H	H	H	OMe	29
2i	H	H	H	NO_2	12
2j	H	H	H	CN	19
2k	H	H	H	$CONH_2$	>100
2l	Ac	H	H	Cl	>100
2m	Bn	H	H	Cl	>100
AEBSF[a]					39

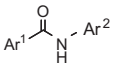
[a] 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride.

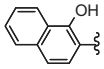
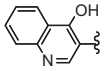
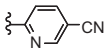
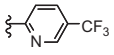
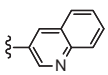
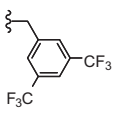
Table 2
Inhibition of TMPRSS4 serine protease activity by 2-hydroxydiarylamides **3a–q**



Compound	R′1	R′2	R′3	R′4	IC_{50} (μM)
3a	H	H	H	H	50
3b	Me	H	Me	H	39
3c	Cl	H	Cl	H	19
3d	CF_3	H	OMe	H	16
3e	CF_3	H	Br	H	12
3f	CF_3	H	H	CF_3	20
3g	CF_3	CF_3	H	H	13
3h	CF_3	Br	H	H	30
3i	CF_3	Cl	H	H	35
3j	CF_3	H	H	F	28
3k	H	CF_3	H	H	16
3l	CF_3	H	H	H	13
3m	H	CN	H	H	76
3n	CF_3	OMe	CF_3	H	12
3o	CF_3	Me	CF_3	H	10
3p	CF_3	Br	CF_3	H	6
3q	Cl	Cl	Cl	H	>100

Table 3
Inhibition of TMPRSS4 serine protease activity by 2-hydroxydiarylamides **2n–p**, **5a–f** and **6**



Compound	Ar^1	Ar^2	IC_{50} (μM)
2n		3,5-bis-CF_3-Ph	>100
2o		3,5-bis-CF_3-Ph	>100
2p		3,5-bis-CF_3-Ph	>100
5a	2-OH-5-Cl-Ph		80
5b	2-OH-5-Cl-Ph		>100
5c	2-OH-5-Cl-Ph		>100
5d	2-OH-5-Cl-Ph		>100
5e	2-OH-5-Cl-Ph		>100
5f	2-OH-5-Cl-Ph		85
6	2-OH-5-Cl-Ph		18

3,5-disubstituted phenyl group (**2a, 3d** and **3e**) produced more effective inhibitors of TMPRSS4 serine protease activity than the 2,5- and 3,4-disubstituted (CF_3–CF_3 or CF_3–X) phenyl groups (**3f–j**). In trisubstituted derivatives (**3n–q**), bis-CF_3-containing compounds (**3n–p**) exhibited strong inhibitory activities compared with mono- (**3k–m**) or disubstituted (**2a** and **3b–j**) derivatives. Notably, compound **3p** (*C*Log*P* = 7.36), with bromine at the R′2 position, displayed two-fold higher inhibition than compound **2a** (*C*Log*P* = 6.47). We observed that compound **3** showed good correlations between inhibitor potency and the lipophilicity (*CLogP*) of the molecule (see *CLogP* values in Table S2).

Table 4
Percent inhibition of invasion and cell viability

Compound	0.1 μM[a]	Cell viability[b]
2a	45	90
2i	26	96
3e	43	93
3o	43	90
3p	53	89

[a] Percent inhibition of invasion compared with DMSO, negative control.
[b] Cell viability was measured by CCK-8 assays, higher value indicates lower cytotoxicity. All biological evaluations were performed at least in triplicate in two independent experiments.

In the study of extending the SAR scope, several 2-hydroxy diarylamides, containing napthtyl, (**2n**), pyridinyl (**2o**) and quinolinyl (**2p**) instead of 2-hydroxy-4-chlorophenyl of compound **2a**, did not inhibit TMPRSS4 serine protease (Table 3). In addition, the introduction of pyridinyl (**5a–d**), pyrimidinyl (**5e**), quinolinyl (**5f**) groups resulted in a significant loss of potency, with the exception of the benzamide **6** (IC_{50} = 18 μM).

Next, select compounds were evaluated for suppressing the invasion of colon cancer cells. SW480 colon cancer cells overexpressing TMPRSS4 (T19) were allowed to invade a reconstituted basement membrane (Matrigel) in the presence of these compounds as previously described.[6]

Compounds **2a**, **3e**, and **3o** moderately inhibited the invasiveness of TMPRSS4-overexpressing colon cancer cells (T19), whereas compound **2i** displayed relatively modest inhibitory activity (Table 4). Compound **3p** displayed the strongest inhibitory activity against the invasiveness of TMPRSS4-overexpressing colon cancer cells (Table 4). These results are consistent with those of in vitro TMPRSS4 serine protease inhibitory activity assays. Notably, relatively high IC_{50} values of inhibitors against TMPRSS4 serine protease activity in vitro compared with those against cancer cell invasion may be partially due to the low specific activity of recombinant TMPRSS4 serine protease protein although we could not completely exclude the possibility of off-target effects of the compounds on cell invasion. A CCK-8 assay[22] confirmed that these compounds did not substantially affect cell viability or growth, which was expected on the basis of the observation that TMPRSS4 did not confer a growth advantage to colon cancer cells.[9] These observations indicate that these derivatives are efficacious in suppressing cancer cell invasion associated with TMPRSS4 serine protease activity without exhibiting non-specific cytotoxicity.

In summary, we have discovered a novel series of 2-hydroxydiarylamide derivatives as potential TMPRSS4 serine protease inhibitors. In our SAR studies, compound **3p** was observed to be the most potent (IC_{50} = 6 μM) inhibitor of TMPRSS4 serine protease with the anti-invasive activity. Molecular modeling studies of the 2-hydroxydiarylamide series are underway and will be reported upon completion.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.01.055.

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10. TMPRSS4 serine protease assay was performed with peptide substrates fluorometrically. Briefly, the TMPRSS4 serine protease domain (amino acid residues 205–437) with 15 amino acids (DYKDDDGDYKDDDDK) containing an enterokinase cleavage site at the N-terminus was subcloned into the expression vector pET21b (Novagen, Darmstadt, Germany) by PCR with the following primer set: (5′-TCATATGGATTACAAA GACGATGACGGTGACTACAA AGATGACGACGATAAGGTGGTGGGTGGGGAGG-3′ and 5′-GAATCTCGAGCAGCT CAGCCTTCCAGACAT-3′). A recombinant TMPRSS4 serine protease domain fused with an enterokinase cleavage sequence (pro-form) with a six times His tag was expressed in *Escherichia coli* BL21(DE3), purified with Ni-NTA resin (Qiagen, Valencia, CA), and treated with enterokinase (New England Biolabs, Ipswich, MA) to produce the active form of recombinant TMPRSS4 serine protease. Enterokinase was removed by purifying the recombinant proteins by Ni-NTA affinity chromatography. To examine the TMPRSS4 proteolytic activity, 1 μg of the active form of recombinant TMPRSS4 serine protease was incubated with 100 μM Z-Phe-Arg-7-amido-4-methylcoumarin hydrochloride (Sigma, St Louis, MO) in a reaction buffer (100 mM Tris, pH 8.0, 10 mM $CaCl_2$, and 1 μM $ZnCl_2$) at 25 °C. The fluorescence resulting from the hydrolysis of the peptide substrate was measured with a fluorometer (Victor3 plate reader, PerkinElmer, Wellesley, MA) using excitation and emission wavelengths of 385 and 455 nm, respectively.
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22. A CCK-8 assay was performed to measure the cytotoxicity of the compounds in TMPRSS4-overexpressing colon cancer cells. Cells were seeded onto 96-well plates at a density of 10,000 cells/100 μl/well and incubated with or without 0.1 μM of each compound at 37 °C. After 2 days of incubation, 10 μl of CCK-8 solution (Dojindo Laboratories, Kumamoto, Japan) was added to each well, and the absorbance at 450 nm was measured. The optical density directly correlated with cell viability.