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To be cited as: *ChemMedChem* 10.1002/cmdc.202100273

Link to VoR: https://doi.org/10.1002/cmdc.202100273

WILEY-VCH

FULL PAPER WILEY-VCH

Structural development of salicylanilide-based SPAK inhibitors as candidate antihypertensive agents

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Abstract: Hypertension is an important target for drug discovery. We have focused on the with-no-lysine kinase (WNK)-oxidative stress-responsive 1 (OSR1) and STE20/SPS1-related proline–alanine-rich protein kinase (SPAK)-NaCl cotransporter (NCC) signal cascade as a potential target, and we previously developed a screening system for inhibitors of WNK-OSR1/SPAK-NCC signaling. Herein we used this system to examine the structure-activity relationship (SAR) of salicylanilide derivatives as SPAK kinase inhibitors. Structural design and development based on our previous hit compound, aryloxybenzanilide derivative **2**, and the veterinary anthelmintic closantel (**3**) led to the discovery of compound **10a** as a potent SPAK inhibitor with reduced toxicity. Compound **10a** decreased the phosphorylation level of NCC in mouse kidney in vivo, and appears to be a promising lead compound for a new class of antihypertensive drugs.

Introduction

Hypertension affects more than one billion people worldwide, and can lead to potentially fatal diseases such as heart attack, stroke, and aneurysmal rupture. Therefore, there is a need for effective antihypertensive agents. To develop such agents, it is important to understand the mechanisms of blood pressure regulation. For example, pseudohypoaldosteronism type II (PHAII) is a monogenic hypertensive disease,[1,2] also known as familial hyperkalemic hypertension or Gordon's disease. PHAII is an autosomal dominant disease characterized by hypertension, severe hyperkalemia, and mild metabolic acidosis,[3] and is associated in at least some patients with mutations in the with-no-lysine kinases WNK1 and WNK4 genes.[4] Specifically, patients with WNK1 mutations have large intronic deletions that increase the abundance of WNK1, and patients with WNK4 mutations have missense mutations localized in a short region near putative coiled-coil domains in a highly conserved sequence of WNK. WNK kinases are so named because of the lack of lysine in the ATP-binding cassette of the catalytic region.

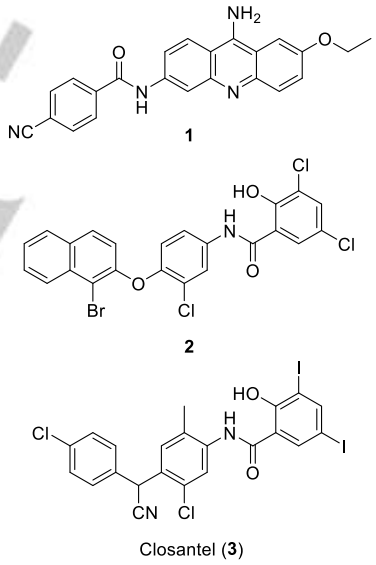


Figure 1. Structures of our developed WNK-OSR1/SPAK binding inhibitor (**1**) and hit compounds **2** and closantel (**3**) discovered by SPAK inhibitor screening.

We previously generated PHAII model mice (Wnk4$^{D561A/+}$ knock-in mice) that carry the same mutation as PHAII patients, and we discovered that constitutive activation of the WNK-oxidative stress-responsive 1 (OSR1) and STE20/SPS1-related proline–alanine-rich protein kinase (SPAK)-NaCl cotransporter (NCC) signal cascade is the major pathogenic mechanism of PHAII.[5,6] WNK-OSR1/SPAK-NCC signaling is constitutively activated in PHAII, and the increased phosphorylation and activation of NCC cause excessive sodium reabsorption in the distal convoluted tubules in the kidneys, resulting in salt-sensitive hypertension. WNK signaling is positively controlled by aldosterone, angiotensin II, and insulin, which may contribute to hypertension in patients with hyperaldosteronism and hyperinsulinemia.[7–11] In addition, WNK-OSR1/SPAK-NKCC1 phosphorylation and activation in vascular smooth muscle cells play a central role in the regulation of vascular tonus.[12-15] Consequently, compounds that inhibit this signal cascade are

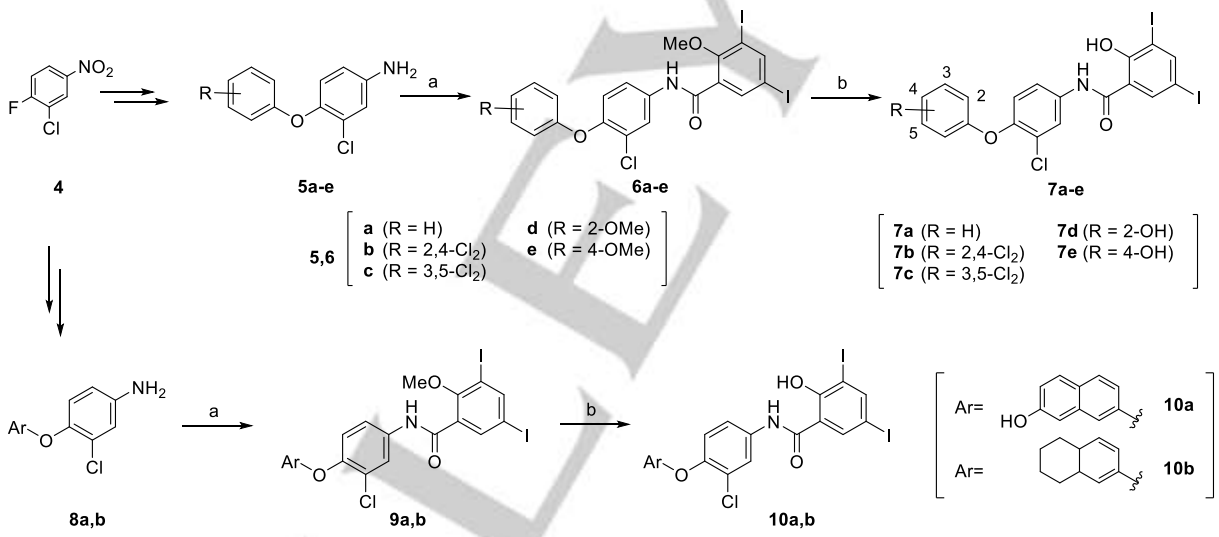
expected to reduce hypertension through dual actions, namely NaCl diuresis and vasodilation, and may be effective to treat patients with hyperaldosteronism or hyperinsulinemia. In addition to hypertension, recent studies have revealed that SPAK inhibitors have therapeutic potential for treating multiple diseases, including neurological diseases,[16,17] inflammatory colitis, and cystic fibrosis.[18] On the basis of these considerations, we have investigated the development of WNK-OSR1/SPAK-NCC signaling inhibitors as a novel class of antihypertensive agents.[19,20] For example, we previously developed inhibitors of WNK binding to OSR1/SPAK, such as **1**.[21] We also developed a screening system for SPAK kinase inhibitors using ELISA, obtaining aryloxybenzanilide derivative **2** as a hit compound,[20] and we recently reported on the structure-activity relationship of **2**.[22] In addition, we found that a veterinary anthelmintic closantel (**3**) exhibited SPAK-inhibitory activity.[20] Interestingly, the core structures of **2** and **3** are similar. However, overdosing of **3** causes various adverse effects, including neurotoxicity.[23-25] In this study, therefore, we investigated the structural development and SAR of salicylanilide-based SPAK inhibitors, focusing on hybrid structures of compounds **2** and **3**, with the aim of finding potent SPAK inhibitors with reduced toxicity, or a more favorable ratio of activity to toxicity.

Results and Discussion

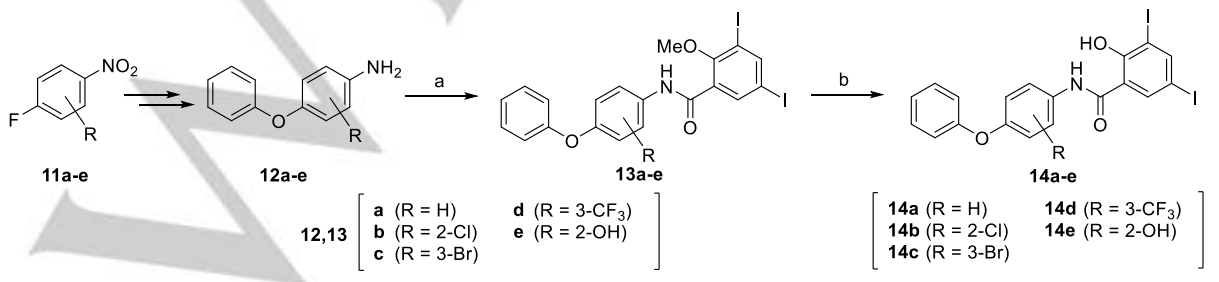
Design and synthesis

Compounds **2** and **3** share a salicylanilide moiety as a common structural motif. Our previous SAR study based on compound **2** also indicated that the 2-hydroxyl group of the benzoyl group is important for the SPAK-inhibitory activity. In addition, closantel (**3**) exhibited inhibitory activity toward NCC phosphorylation *in vivo*.[20] Therefore, we considered that the 3,5-diiodosalicylanilide structure is a promising pharmacophore for SPAK inhibitors. Here, we designed and synthesized a series of 3,5-diiodosalicylanilide derivatives as hybrid structures based on benzanilide **2** and closantel (**3**), and compared them with 3,5-dichlorosalicylanilide derivatives.

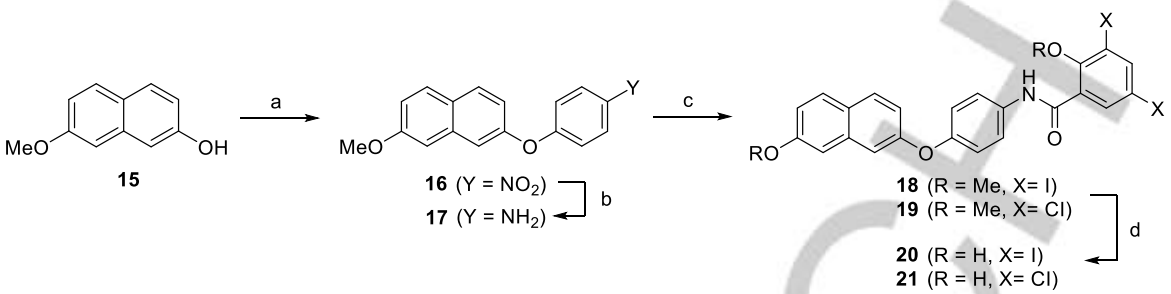
Synthesis of the 3,5-diiodosalicylanilide derivatives is shown in Schemes 1, 2 and 3. Scheme 1 shows the synthesis of compounds **7** and **10** bearing various aryloxy moieties. Anilines **5a**-**e** were prepared from compound **4** as described in our previous paper (Figure S1).[22] Condensation between anilines **5a**-**e** and 3,5-diiodo-2-methoxybenzoic acid afforded compounds **6a**-**e**, and the removal of the *O*-methyl group by boron tribromide afforded the designed compounds **7a**-**e**, respectively. Compounds **10a** and **10b**, bearing a bicyclic group as the aryloxy moiety, were similarly prepared (Scheme 1).



Scheme 1. Reagents and conditions: (a) 3,5-diiodo-2-methoxybenzoic acid, EDC, HOBt, DMF, 40 °C, 60%-quant.; (b) BBr_3, CH_2Cl_2, rt, 37%-88%

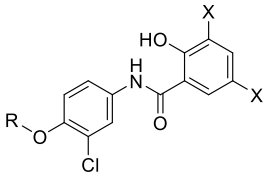


Scheme 2. Reagents and conditions: (a) phenol, K_2CO_3, DMF, rt, 97%-quant.; (b) Fe powder, HCl, EtOH- H_2O, reflux, 72%-94%.; (c) 3,5-diiodo-2-methoxybenzoic acid, EDC, HOBt, DMF, 40 °C, 60%-quant.; (d) BBr_3, CH_2Cl_2, rt, 37%-88%.



Scheme 3. Reagents and conditions: (a) 4-fluoronitrobenzene, K_2CO_3, DMF, rt, 97%; (b) Fe powder, HCl, EtOH-H_2O, reflux, 72%; (c) method A: 3,5-dichloro-2-methoxybenzoic acid, $(COCl)_2$, DMF, CH_2Cl_2, rt, then **20**, Et_3N, THF, rt.; method B: 3,5-diiodo-2-methoxybenzoic acid, EDC, HOBt, DMF, 40 °C; (d) BBr_3, CH_2Cl_2, rt, 37%-88%.

Table 1. Biological activity of salicylanilide derivatives **7**, **10**, **22** and **23**.



Compound	R =	X	ELISA IC_{50} (μM)	mpkDCT activity[b]	mpkDCT CC_{50} (μM)	CC_{50}/IC_{50}	Log*P*[e]
2	1-Br-naphth-2-yl	Cl	0.26[a]	+++	2.0	7.7	7.49
Closantel (**3**)	—	—	0.77	+++	7.9	10	8.50
7a	Ph	I	2.6	+	n.t.[c]	n.d.[d]	7.26
7b	2,4-Cl_2−C_6H_3		1.0	+	n.t.[c]	n.d.[d]	8.38
7c	3,5-Cl_2−C_6H_3		0.90	+	3.3	3.7	8.38
7d	2-OH−C_6H_4		4.2	n.t.[c]	9.8	2.3	6.87
7e	4-OH−C_6H_4		4.2	+	13	3.1	6.87
10a	7-OH-naphth-2-yl		0.30	+++	8.3	28	7.87
10b	tetrahydronaphth-2-yl		1.6	-	3.2	2.0	8.57
22	4-OH−C_6H_4	Cl	5.6[a]	-	7.2	1.3	5.27
23	7-OH-naphth-2-yl		5.1[a]	+++	7.3	1.4	6.27

[a] Taken from our previous report.[22] [b] Activity toward mpkDCT cells is indicated as follows: +++, IC_{50} value between 1.6 μM and 3.2 μM; +, IC_{50} value between 6.3 μM and 13 μM; -, IC_{50} value larger than 25 μM or no significant activity. [c] Not tested, [d] Not determined. [e] Calculated with ChemDraw15.

Synthesis of compounds **14** bearing various substituents on the central benzene ring is shown in Scheme 2. These compounds were synthesized in a similar manner to that described for the preparation of compounds **7**. Namely, condensation between 3,5-diiodo-2-methoxybenzoic acid and anilines **12a-e** prepared from **11a-e** gave **13a-e**, and the removal of the *O*-methyl group afforded the designed compounds **14a-e**, respectively (Scheme 2). Compounds **20** and **21** are derivatives of compound **10a** without the chlorine atom, and were synthesized similarly from **15** as a starting material (Scheme 3). Synthesis of 3,5-dichlorosalicylanilide derivatives **22**, **23** and **24a-d** was described in our previous report.[22]

Biological activity

The SPAK-inhibitory activity of the synthesized compounds was assessed by means of our previously developed ELISA. The ELISA plate was coated with GST-NKCC2 [1–174] and the plate was incubated with GST-SPAK [T233E] in the presence of GST-MO25a. NKCC2 phosphorylation was detected by anti-p-NKCC2 (pThr100/105) antibodies.[20] We further investigated the inhibitory effect on NCC phosphorylation using a cell-based assay with mouse renal distal tubule-derived (mpkDCT) cells, which endogenously express NCC. We also examined cytotoxicity with the same cell line.

First, we examined the structure-activity relationship of the aryloxy group at the left terminal of compounds **2** (Table 1). Compound **7a** without any substituent on the phenoxy group exhibited moderate inhibitory activity with an IC_{50} value of 2.6 μM in ELISA. Introduction of two chlorine atoms (**7b** and **7c**) enhanced the activity, but **7c** exhibited higher cytotoxicity than **3** toward mpkDCT cells, resulting in a lower CC_{50}/IC_{50} ratio than that of **3**. Though introduction of a hydroxyl group (**7d** and **7e**) reduced the cytotoxicity, the SPAK-inhibitory activity of **7d** and **7e** were less than the lead compounds, resulting in low CC_{50}/IC_{50} ratio. The corresponding 3,5-dichlorosalicylanilide

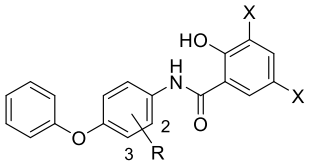
derivative **22** bearing a 4-hydroxyphenyl group also exhibited a low CC_{50}/IC_{50} ratio. These results indicated that introduction of the hydrophilic functionality reduces the cytotoxicity, but the decrease of hydrophobicity is unfavorable for inhibitory activity. On the basis of these considerations, we investigated the activity and toxicity of hydroxynaphthyl derivative **10a**, and found that it exhibited potent SPAK-inhibitory activity *in vitro* (IC_{50}; 0.30 μM), as well as a significant inhibitory effect on NCC phosphorylation in mpkDCT cells, and an improved CC_{50}/IC_{50} ratio. The corresponding 3,5-dichlorosalicylanilide derivative **23** also exhibited inhibitory activity in mpkDCT cells, though its CC_{50}/IC_{50} ratio was significantly smaller than that of **10a** (Table 1).

Next, we investigated the structure-activity relationship of the central benzene moiety (Table 2). Removal of the chlorine atom of compound **7a**, yielding compound **14a**, increased the inhibitory activity (IC_{50} 1.7 μM in ELISA assay). Introduction of a bromine (**14c**), trifluoromethyl (**14d**) or hydroxyl group (**14e**) enhanced the inhibitory activity in ELISA assay, but these compounds did not inhibit NCC phosphorylation in mpkDCT cells. Similar results were observed with the 3,5-dichlorosalicylanilide derivatives **24**, namely, compounds bearing a bromine (**24b**), trifluoromethyl (**24c**) or hydroxyl group (**24d**) exhibited more potent activity than 3-chloro derivative **24a**, but did not inhibit NCC phosphorylation in mpkDCT cells.

Since compound **14a** without a chlorine atom on the central benzene ring showed higher potency than 3-chloro derivative **7a**, we investigated the importance of the chlorine atom of the 7-hydroxynaphthoxy derivative **10a**, which exhibits potent activity and a large CC_{50}/IC_{50} value. Table 3 shows the biological activity of the 7-hydroxynaphthoxy derivatives. In contrast to the phenoxy derivatives shown in Table 2, compound **20** lacking the chlorine atom was significantly less potent than 3-chloro derivative **10a**, and did not inhibit NCC phosphorylation in mpkDCT cells. The corresponding 3,5-dichlorosalicylanilide derivative **21** also showed no inhibitory activity in mpkDCT cells. These results indicated that the 3-chloro substitution is essential for the inhibitory activity of the 7-hydroxynaphthoxy derivatives in mpkDCT cells, in contrast to the result for **14a**.

We next examined NCC phosphorylation in mouse kidney using closantel (**3**) as the positive control in order to establish whether compound **10a** is effective *in vivo*. As shown in Figure 2, phosphorylation level of NCC were decreased in the kidneys at 30 minutes after intraperitoneal injection of **10a** (20 mg/kg), and had recovered by 100 minutes after administration. These results suggest that **10a** inhibited SPAK kinase *in vivo*, and the inhibition was reversible. We previously reported that the SPAK-inhibitory activity of closantel were short-acting and reversible.[20] Thus, our findings indicate that **10a** functions as a SPAK inhibitor in the same manner as closantel, but has an improved activity-toxicity profile.

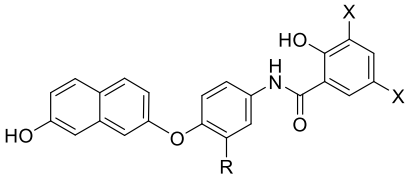
Table 2. Biological activity of 3,5-diiodosalicylanilide derivatives **14a**-**14e** and 3,5-dichlorosalicylanilide derivatives **24a**-**24d**.



Compound	R =	X	ELISA IC_{50} (μM)	mpkDCT activity[b]	mpkDCT CC_{50} (μM)	CC_{50}/IC_{50}	Log*P*[e]
Closantel (**3**)	—	—	0.77	+++	7.9	10	8.50
7a	3-Cl	I	2.6	+	n.t.[c]	n.d.[d]	7.26
14a	H		1.7	+++	2.5	1.5	6.70
14b	2-Cl		5.4	+++	4.1	0.8	7.26
14c	3-Br		0.60	-	2.2	3.7	7.53
14d	3-CF_3		0.70	-	2.0	2.9	7.62
14e	2-OH		0.90	-	7.3	8.1	6.31
24a	3-Cl	Cl	4.5[a]	+++	2.8	0.6	5.66
24b	3-Br		1.5[a]	-	7.2	4.8	5.93
24c	3-CF_3		2.4[a]	-	5.9	2.5	6.02
24d	2-OH		2.1[a]	-	7.3	3.5	4.71

[a] Taken from our previous report.[22] [b] Activity toward mpkDCT cells is indicated as follows: +++, IC_{50} value between 1.6 μM and 3.2 μM; +, IC_{50} value between 6.3 μM and 13 μM; -, IC_{50} value larger than 25 μM or no significant activity. [c] Not tested, [d] Not determined. [e] Calculated with ChemDraw15.

Table 3. Biological activity of 7-hydroxynaphthoxy derivatives **10a**, **20**, **21** and **23**.



Compound	R =	X	ELISA IC_{50} (μM)	mpkDCT activity[b]	mpkDCT CC_{50} (μM)	CC_{50}/IC_{50}	Log*P*[e]
Closantel (**3**)	—	—	0.77	+++	7.9	10	8.50
10a	Cl	I	0.30	+++	8.3	28	7.87
20	H		2.5	-	7.6	3.0	7.31
21	H	Cl	3.0	-	6.3	2.1	5.71
23	Cl		5.1[a]	+++	7.3	1.4	6.27

[a] Taken from our previous report.[22] [b] Activity toward mpkDCT cells is indicated as follows: +++, IC_{50} value between 1.6 μM and 3.2 μM; -, IC_{50} value larger than 25 μM or no significant activity. [c] Not tested, [d] Not determined. [e] Calculated with ChemDraw15.

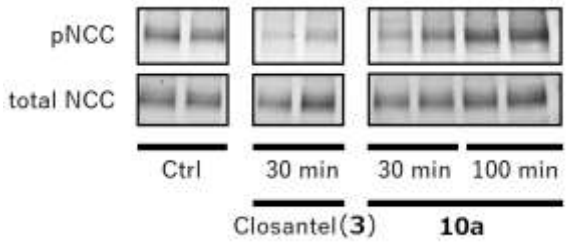


Figure 2. Immunoblots of total NCC and phosphorylated NCC in C57BL/6 mouse kidney after intraperitoneal injection of closantel and compound **10a** (20 mg/kg, i.p.). NCC phosphorylation (p-Ser71) was decreased relative to total NCC in the kidneys at 30 minutes after injection of **10a** and had recovered by 100 minutes after administration.

Conclusion

In this study, we investigated the structure-activity relationship (SAR) and structural development of salicylanilide derivatives as SPAK kinase inhibitors, focusing on hybrid structures designed on the basis of lead compounds **2** and **3**, with the aim of finding a potent inhibitor of WNK-OSR1/SPAK-NCC signaling that shows an improved toxicity profile. Specifically, we designed and synthesized a series of 3,5-diiodosalicylanilide derivatives as candidate SPAK inhibitors. Among them, we discovered compound **10a** as a potent SPAK inhibitor with an improved activity-toxicity profile compared to the lead compounds. Compound **10a** reduced the phosphorylation level of NCC in mouse kidney *in vivo*. It showed short-acting and reversible inhibition, like closantel (**3**). Compound **10a** appears to be a promising candidate for a new class of antihypertensive drugs.

Acknowledgements

This research was supported by Japan Agency for Medical Research and Development (AMED) under Grant Number JP20am0101098 (Platform Project for Supporting Drug Discovery and Life Science Research, BINDS).

Keywords: antihypertensive drugs • hypertension • salicylanilide • SPAK • WNK

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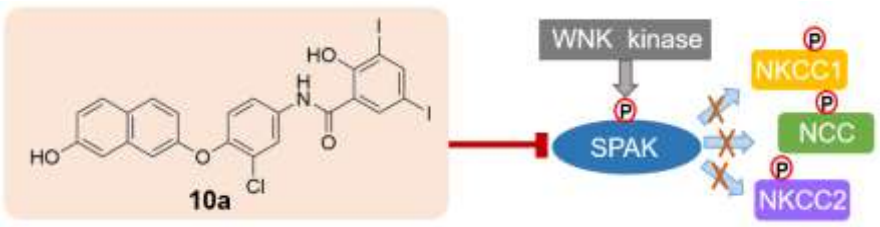

We developed novel salicylanilide derivative **10a** as a potent SPAK inhibitor with an improved activity-toxicity profile. Compound **10a** reduced the phosphorylation level of NCC in mouse kidney *in vivo*. Compound **10a** appears to be a promising candidate for a new class of antihypertensive drugs.